The Dissertation Committee for Jennifer Rebecca Jones Certifies that this is the approved version of the following dissertation:

Novel Roles of the Proteins Oskar and Bluestreak in Germ Cell Formation and Migration

Committee:

Paul Macdonald, Supervisor

Karen Browning

Janice Fischer

Marty Shankland

John Sisson

David Stein

Novel Roles of the Proteins Oskar and Bluestreak in Germ Cell Formation and Migration

by

Jennifer Rebecca Jones, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August, 2007

Dedication

To my parents,

who have supported me throughout my academic career with love and encouragement,

To my sister,

who has always laughed and cried with me,

And to my husband,

who always believes in me.

Acknowledgements

I would like to thank my supervising professor, Dr. Paul Macdonald, for sharing his expertise in research, and his unending patience throughout my graduate career. I would also like to thank the members of my committee, Drs. Karen Browning, Janice Fischer, Marty Shankland, John Sisson and David Stein, for their advice and help during my research. I would like to express gratitude to the members of the Macdonald lab, not only for their help and discussions concerning my research, but also for their friendship over the years. Lastly, I would like to thank my entire family, for their encouragement and love.

Novel Roles of the Proteins Oskar and Bluestreak in Germ Cell Formation and Migration

Publication No._____

Jennifer Rebecca Jones, Ph.D.

The University of Texas at Austin, 2007

Supervisor: Paul M. Macdonald

The formation of germ cells in *Drosophila melanogaster* is dependent on the presence of ribonucleoprotein complexes called polar granules. A key component of these complexes is Oskar, a novel protein which has been shown to nucleate the granules. To investigate whether Oskar plays a further role in polar granule formation, I cloned the *oskar* gene from *D. immigrans* flies (osk^{imm}) and introduced it into *D. melanogaster* flies using P-element transformation. I found that osk^{imm} was able to rescue both the posterior patterning and germ cell formation defects of embryos from *oskar* mutant mothers. In addition, I found that the polar granules of embryos containing only Osk^{imm} as a source of Oskar protein resemble those found in *D. immigrans* embryos, indicating a new role for Oskar in determining the morphology of the polar granules. Germ cell formation in *Drosophila* is succeeded by migration of the germ cells to the site of gonad formation. A second line of research presented in this dissertation describes analysis of a novel protein important for both germ cell formation and migration, Bluestreak (Blue). Embryos from

either heterozygous or homozygous *Blue*$^{-}$ mothers display defects in germ cell number and shape. I found that the ovaries of *Blue*$^{-}$ females have defects in the localization of Staufen and Oskar, sufficient to cause a reduction in pole cell number in embryos. In addition, genetic analysis of the interaction between Bluestreak and mutants which affect pole cell migration implicates Bluestreak in this process. Finally, I found that Blue localizes to centrosomes along with γ-tubulin throughout the embryo, and to the nuclear membrane in pole cells. My findings introduce the possibility that Bluestreak may act to regulate germ cell migration in *Drosophila*.

Table of Contents

List of Tables

List of Figures

Chapter One: Introduction

GENERAL INTRODUCTION

This thesis describes work regarding the polar granules and pole cells of *Drosophila melanogaster*. Polar granules are large ribonucleoprotein complexes formed at the posterior pole of the *Drosophila* oocyte. The polar granules remain at the posterior pole in the fertilized, freshly laid egg, and are incorporated into the first cells that form within the embryo. These cells are termed pole cells, and are the germ cells of the *Drosophila* embryo. The pole cells will migrate through the gastrulating embryo and eventually coalesce with somatic cells to form the gonads of the mature embryo.

The polar granules are widely accepted as necessary for the formation of the pole cells, since it is their localization in the embryo that corresponds to the formation of germ cells in the embryo (Ephrussi and Lehmann, 1992). In addition, mothers mutant in genes which encode polar granule components produce embryos that lack both granules and pole cells (Boswell and Mahowald, 1985, Harris and Macdonald, 2001, Hay et al., 1988, Lehmann and Nüsslein-Volhard, 1986). Despite a recognized role in pole cell formation, the precise function of the polar granules in the germ cells remains a mystery. In particular, the novel protein Oskar, which is required to nucleate the granules, has no other known function.

In many species, the germ cells of the organism are formed far from the site where the gonads will form in the mature organism. Recently, several genes have been identified in *Drosophila* which control the migration of the pole cells to the somatic gonadal tissue in the mature embryo (reviewed in Renault, 2006, Santos, 2004a). While these studies have elucidated much about the cues necessary for germ cell migration, less is known about the machinery inside the pole cells which allows for efficient migration to the gonad.

The two major aims presented in this thesis relate to polar granules and the proper formation and migration of pole cells. The first aim is to determine what role Oskar might play beyond its known nucleation role in the granules. The second aim is to understand what role a novel protein called Bluestreak plays in pole cell formation and migration.

PRIMORDIAL GERM CELL SPECIFICATION IN MANY SPECIES DEPENDS ON A LOCALIZED GERM PLASM

All multicellular organisms consist of two types of cells, somatic and germline cells. Somatic cells make up the bulk of the cells in an organism, but they also die with the organism. Germline cells will differentiate only into sperm and egg, and therefore have the opportunity to pass on genetic information to the next generation. As carriers of genetic information from one generation to the next, the germline can be considered immortal. In addition, germline cells are pluripotent, meaning they retain the capacity to become any cell in the body of the progeny. It is therefore interesting to determine how germline cells are set aside from the somatic cells of the organism and how these cells maintain this specification and pluripotency.

The idea that primordial germ cells (PGCs) might remain a separate, undifferentiated lineage within an organism was first proposed by Owen in 1849 (Recorded by Wilson, 1896). He believed that not all the cells that arise from the zygote contributed to the body, but that a subset of cells remained unchanged and contributed to the development of the next generation. Subsequent studies by Weismann on the germ cells of hydroids extended this idea by concluding that it was not the germ cells themselves that were continuous between generations, but instead a substance in the germ cells of the parent which is passed on to those of the new individual. While his ideas on the continuity of germ plasm actually referred to the genetic material of an organism, his

hypothesis set the framework for germ plasm theory well into the 20th century (Eddy, 1975).

Aiding the early theory and study of germ plasm was the fact that the eggs of many invertebrates contain a region of cytoplasm that is visibly different from the rest of the cytoplasm of the egg as viewed under the light microscope (Hegner, 1914). The work of several investigators in the early 1900's showed that this cytoplasm, termed germ plasm, was incorporated into the PGCs of several species. Kahle working on the fly *Miastor* was able to follow germ cells from one generation to the next by virtue of the presence of what he termed "polares plasma" in the egg and in the germ cells. Hegner later showed that removing this germ plasm from the eggs of beetles resulted in adults lacking a germline, and extended these studies by showing this effect also occurred in several other invertebrate species (Hegner, 1914). He was able to show that the visible substance in the cytoplasm was present in the egg and germ cells until the germ cells form oogonia and spermatogonia, and then reappeared in the eggs of the next generation. He proposed the existence of "granules" solely in the polar plasm, and that these granules might be the germ cell determinant.

The existence of germ plasm in *Drosophila melanogaster* (pole plasm) was confirmed by several investigators by the early 20th century (see Eddy, 1975). The *Drosophila* embryo develops from the fertilized egg through a series of rapid nuclear divisions, creating a multinucleate syncytium (Campos-Ortega, 1985). By light microscopy, the pole plasm is seen as a region of clear cytoplasm just under the posterior cortex of the egg. Using basic dyes, particles known as polar granules are visible within the pole plasm (Counce, 1963, Mahowald, 1971b). PGCs (called pole cells in *Drosophila*) are formed as the nuclei of the syncytium approach the posterior end of the embryo and are pinched off into cells, incorporating the pole plasm and the granules

within it. In 1931, Geigy showed that UV-irradiation of the pole plasm of the embryo resulted in sterile adult flies (Geigy, 1931). However, it was the transplantation studies performed by Illmensee and Mahowald which showed that pole plasm was sufficient for pole cell formation (Illmensee and Mahowald, 1974, Illmensee and Mahowald, 1976). Pole plasm transferred to ectopic sites in the embryo resulted in the formation of ectopic pole cells, which were shown to be functional for forming adult gonads. However, these studies did not address whether the entire polar plasm or components within the cytoplasm, such as polar granules, resulted in the formation of pole cells (see below).

Mahowald was the first to describe the fine structure of the polar granules of *D. melanogaster* embryos by electron microscopy (Mahowald, 1962, Mahowald, 1968, Mahowald, 1971a). He characterized the granules as fibrous, electron-dense spheres, between 0.2 and 0.5 μm in diameter, without a limiting membrane. As the embryo begins gastrulation, the granules fuse to create larger spheres, up to 1.0 μm in diameter, with an electron-lucid core. By studying the embryos of several different *Drosophila* species, Mahowald also showed that the granules take on species-specific differences in shape and size as they fuse (Mahowald, 1968). Most notably, the polar granules of *Drosophila immigrans* embryos are found as one large oblong granule per cell, as opposed to the smaller, spherical, individual granules of *D. melanogaster*, which are found several per cell (Mahowald et al., 1976). Following fusion, the polar granules of all *Drosophila* species begin to fragment and associate with the outer nuclear envelope of each pole cell, resembling the nuage seen in adult ovaries (Mahowald, 1971a). Nuage is a structure associated with the nuclear envelope in the germ cells of many species, from worms to mammals (for a review, see Eddy, 1975). It is of special interest as nuage is strikingly similar to polar granules in fine structure.

In *Caenorhabditis elegans*, zebrafish and *Xenopus,* specification of the germ line also requires a specialized germ plasm in the egg that is characterized by large electron-dense particles (referred to as germinal granules or P granules, Knaut et al., 2000, Komiya, 1995, Seydoux, 2001). While in species such as mice and humans, germ cells form as a result of inductive interactions between different tissues of the zygote, nuage has still been reported in the ovaries and testis (Eddy, 1996, Extavour and Akam, 2003). The functional significance of the nuage in species such as mice and humans is still unknown.

POLAR GRANULES ASSEMBLE THE GERM PLASM IN *DROSOPHILA*

Mutations which affect germ cell formation in *Drosophila* have been dubbed "grandchildless" mutations, since embryos from homozygous mutant mothers lack pole cells. These maternal effect mutants, in which the genotype of the mother determines the presence of germ cells in the embryo, regardless of the genotype of the father, revealed that formation of germ cells is initiated by genes expressed during oogenesis. Large scale screens for maternal effect mutations in *Drosophila* have identified genes which affect the formation of pole cells (Boswell and Mahowald, 1985, Lehmann and Nüsslein-Volhard, 1986, Schupbach, 1989). These genes are also members of the "posterior group", so named because mutations not only affect the formation of germ cells, but embryos from homozygous mutant mothers also lack posterior abdominal segments (St Johnston, 1993). So far, genetic screens have identified several genes involved in pole plasm formation: *vasa, tudor, oskar, aubergine, valois, cappuccino, staufen, spire, orb, homeless, pipsqueak, tropomyosin II and mago nashi* (Rongo and Lehmann, 1996, Wilson et al., 1996). Two further posterior group genes, *nanos (nos)* and *pumillo (pum)*,

do not affect germ cell formation, but rather affect posterior patterning alone. The connection between *nos, pum,* and the other posterior group genes is discussed below.

Germ plasm formation begins in the ovary, which is made up of several ovarioles. Ovarioles are strings of increasingly older egg chambers, each egg chamber being made up of 16 germ cells - one oocyte and 15 nurse cells. Both the oocyte and nurse cells of each egg chamber are surrounded by a layer of somatic cells termed follicle cells (King, 1970). The nurse cells will provide the developing oocyte with the RNAs and proteins required for development. The gene products of grandchildless genes are produced in the nurse cells and then localized to the posterior pole of the oocyte (Rongo and Lehmann, 1996). The first component of the pole plasm detectable in oocytes is *oskar* (*osk*) mRNA, which is found throughout the oocyte from the germarium onwards, and localizes to the posterior pole in stage 8 oocytes (Kim-Ha et al., 1991). This localization is dependent on sequences within the *osk* 3'UTR (Kim-Ha et al., 1993). Mutations in *staufen, cappuccino, spire, mago nashi, tropomyosin II* and *orb* also affect this localization (Christerson and McKearin, 1994, Erdélyl et al., 1995, Kim-Ha et al., 1991, Lantz et al., 1994, Newmark and Boswell, 1994).

Polar granules are nucleated by Oskar protein

Upon localization to the posterior pole, *osk* mRNA will begin to be translated during stage 9 of oogenesis, and recruit Vasa, Aubergine and Tudor proteins to the posterior (Breitwieser et al., 1996, Ephrussi and Lehmann, 1992, Harris and Macdonald, 2001, Kim-Ha et al., 1995). Only these four proteins - Oskar (Osk), Vasa (Vas), Aubergine (Aub), and Tudor (Tud) (along with *pgc* RNA, see below) - have been shown to be concentrated in polar granules (Bardsley et al., 1993, Breitwieser et al., 1996, Harris and Macdonald, 2001, Hay et al., 1988, Lasko and Ashburner, 1990). A key role for Osk

was determined through experiments by Ephrussi and Lehmann (1992). They took advantage of the fact that *bicoid* (*bcd*) mRNA is localized to the anterior via its 3'UTR (Macdonald and Struhl, 1988) and that *osk* mRNA localization is dependent on sequences in its 3'UTR (Kim-Ha et al., 1993). They created a transgene with the *osk* 5'UTR and coding sequences and the *bcd* 3'UTR to mislocalize *osk* mRNA to the anterior of embryos. They found that in these embryos, pole cells were formed both at the posterior, due to the action of endogenous Osk, and at the anterior, presumably due to the *osk-bcd3'UTR* transgene. They further found that all of the known polar granule components were recruited to the anterior, and that the pole cells that form there are functional germ cells (Ephrussi and Lehmann, 1992, Megosh et al., 2006, Nakamura et al., 1996). No other polar granule or pole plasm component shares this property, indicating that Oskar alone is capable of nucleating the polar granules. Furthermore, this experiment demonstrates that Osk is sufficient to assemble the germ plasm and form functional pole cells. By assessing the effects of the posterior group mutations in the *osk-bcd3'UTR* background, Ephrussi and Lehmann (1992) were able to place the posterior group genes within a hierarchy. One group of genes was placed upstream of *oskar*, presumably involved in its localization and translational control (*cappuccino, spire, staufen*), since maternal mutations did not affect anterior pole cell formation. *vasa* and *tudor* were placed downstream, since the formation of pole cells was perturbed in these mutants.

In addition to anterior pole cells, the embryos of *osk-bcd3'UTR* mothers are bicaudal, meaning that they have posterior features duplicated at what would be the anterior of the embryo. It was found in these embryos that the posterior determinant, Nanos (Nos), was also directed to the anterior via Osk (Ephrussi and Lehmann, 1992), indicating that Osk not only organizes the pole plasm, but is integral for posterior patterning. It has been shown since that *nos* mRNA must be localized to the posterior

pole via the organization of the pole plasm for efficient translation and that this translational regulation is mediated by sequences in the *nos* 3'UTR (Gavis and Lehmann, 1994, Gavis et al., 1996). As posterior patterning determinants, *nos,* together with *pum,* have been shown to repress the translation of the anterior morphogens *bcd* and *hb* (Murata and Wharton, 1995).

The precise function of the polar granules is still unknown

While it has been shown that the polar granules are necessary and sufficient for pole cell formation, the exact role of the granules in this process is still unknown. Of the known components of the polar granules, only two, Vas and Aub, have distinct conserved functions that have been assessed. Vas is a DEAD-box helicase, which has been shown to bind and unwind RNA in vitro (Liang et al., 1994). Vas also shows sequence similarity to initiation factor eIF4A, which itself is a helicase involved in 5' cap recognition and the binding of RNA to ribosomes (Hay et al., 1988, Lasko and Ashburner, 1988, Rhoads, 1993). Aub contains PIWI and PAZ domains, and has been shown to bind rasiRNAs (repeat-associated-siRNAs), which have been implicated in retrotransposon and stellate silencing in the germline (Aravin, 2004, Cerutti et al., 2000, Harris and Macdonald, 2001, Lin, 2007). Interestingly, both Vas and Aub, (along with Tud, below) are also shared components between polar granules and nuage in *Drosophila*. In fact, homologs of Vas have been identified in both invertebrates and vertebrates (Raz, 2000). In those species where Vas localization has been determined, it has consistently been found to associate with germinal granules and/or nuage in germ cells.

The functions of Tud and Osk are still unknown. Tud contains 11 Tudor domain repeats, which are found in a variety of proteins in many different organisms. Recent

studies indicate that the Tudor domain is a protein-protein interaction domain which binds to proteins which have been symmetrically methylated (Kim, 2006, Maurer-Stroh, 2003). Embryos from *tud* mutant mothers still produce polar granules, however they are smaller and fewer in number, raising the possibility of a role for Tudor in the fusion of granule material (Boswell and Mahowald, 1985, Thomson and Lasko, 2004). We show here, that beyond the role of nucleating the granules, Osk plays a role in determining the ultimate morphology of the mature granules in gastrulating embryos (Chapter 2, this work). We suggest that Osk may act in concert with Tudor to determine this morphology.

While the identification of further polar granule components might better elucidate their function, both genetic and biochemical approaches present complications. Previous genetic screens were conducted to saturating levels, suggesting that the isolation of new mutants would be rare and therefore require large numbers to obtain new mutants. In addition, these screens must be carried out to the F_3 generation in order to access the "grandchildless" phenotype of homozygous mutant mothers. Extensive efforts to biochemically purify polar granules have been less successful than genetic screens in identifying further components, though, *mitochondrial large rRNA (mtlrRNA)* and *polar granule component (pgc)* have been identified (Kobayashi and Okada, 1989, Waring et al., 1978). Kobayashi and Okada (1989) identified *mtlrRNA* as a polar granule component, since it is capable of restoring the ability to form pole cells in UV irradiated embryos. While further studies went on to show that *mtlrRNA* localizes to the edge of polar granules prior to pole formation (Kashikawa et al., 2001), the idea that *mtlrRNA* is a crucial component is still suspect, as pole cells formed in the UV-irradiated embryos are not functional germ cells (Kobayashi and Okada, 1989). Furthermore, studies of polar granules across many *Drosophila* species show that the granules do not necessarily associate with mitochondria (as they do in pre-pole cell *D. melanogaster* embryos),

indicating that the association of *mtlrRNA* with the granules may not be necessary (Mahowald, 2001).

The identification of *pgc* has elucidated an important property of pole cells, transcriptional quiescence (Deshpande, 2004). As soon as the germ cells begin to bud, the germ cell nuclei become transcriptionally repressed (Zalokar, 1976). *pgc* RNA is an untranslated RNA that was discovered using mRNA differential display of wildtype and *tud* embryos (Nakamura et al., 1996), and was further shown to be necessary for transcriptional silencing in germ cells (Deshpande, 2004, Martinho et al., 2004). In embryos from *pgc* mutant females, several transcripts are found in pole cells that are normally only transcribed in the surrounding somatic cells (Martinho et al., 2004). Germ cells also fail to migrate properly and many germ cells die precociously. Similar phenotypes have also been observed in embryos from *nos* and *pum* mutant mothers, which are incidentally also required for transcriptional quiescence (Asaoka-Taguchi, 1999). *gcl* has also been implicated in transcriptional repression in the germ cells. Gcl localizes perinuclearly and mutants in *gcl* display a reduction in germ cell number (Jongens et al., 1994). While transcriptional silencing is normal in *gcl* mutant germ cells, ectopic expression of *gcl* interferes with transcription, suggesting that transcriptional silencing of the posterior nuclei via Gcl acts as a prerequisite for germ cell formation (Leatherman et al., 2002).

GERM CELLS MUST MIGRATE THROUGH THE EMBRYO DURING GASTRULATION

A common property of PGCs in most species is motility, since the germ cells are formed far from the site of the mature gonad. The somatic cells of the embryo control the pathway of migration (Godin, 1990, Santos, 2004a), while germ cells reach the somatic gonadal precursors (SGPs) through a combination of passive morphogenetic movements

and active migration. In the chicken embryo, germ cells are incorporated into the developing blood islands and are then carried through the early circulatory system before exiting in the region of the gonad (Nieuwkoop, 1979). In *Drosophila*, *Xenopus*, and mouse embryos, germ cells are incorporated into the forming hindgut, through which they actively migrate to join the SGPs (Chiquione, 1954, Gomperts, 1994, Sonnenblick, 1941).

Insights on pole cell migration in *Drosophila* by direct visualization of fixed embryos

Several early investigators described the process of pole cell migration according to morphological criteria in fixed embryos (Poulson, 1950, Rabinowitz, 1941, J Morphol, 69, 1-49, Sonnenblick, 1941, Sonnenblick, 1950). These studies laid the groundwork for understanding the formation and migration of the pole cells, however, questions remained unanswered. In particular, it was still uncertain whether the pole cells contributed only to the germline (Poulson, 1950). Later studies used pole cells labeled in various ways specifically to determine the fate of the pole cells (Hay et al., 1988, Jaglarz, 1994, Technau and Campos-Ortega, 1986, Underwood et al., 1980). The least invasive of these was the work of Hay et al. (1988), which fortuitously discovered an antibody that localizes specifically to the pole cells. Using this antibody to follow and quantitate the germ cells throughout migration, Hay and others were able to show that while some pole cells are lost and die during migration, pole cells contributed only to the germline.

The path of pole cell migration occurs as follows. Pole cells first form as a small group of cells at the posterior pole of the syncytial blastoderm. They are the first cells to cellularize after the arrival of nuclei at the end of the syncytial blastoderm stage, though the trigger for their cellularization is not the nuclei, but centrioles (Raff, 1989). The pole cells remain at the posterior pole as the rest of the embryo cellularizes. Shortly after

cellularization, the embryo begins gastrulation and the primordial midgut epithelium (PMG) invaginates dorsally and anteriorly into the embryo. The pole cells are passively carried as a group into the embryo by the PMG, although they begin to show signs of migratory behavior (formation of pseudopodia) at this stage (Jaglarz and Howard, 1995). At the end of germ band elongation, the pole cells actively and individually migrate through the PMG epithelium, presumably due to changes in this epithelium which allow the pole cells to pass through (Callaini, 1995, Jaglarz and Howard, 1995). Subsequent to this transepithelial migration, the pole cells migrate both anteriorly and dorsally, bifurcate, and finally migrate towards the SGPs. At this point the pole cells will coalesce and become encapsulated by the SGPs (for a review, see Starz-Gaiano and Lehmann, 2001).

Genetic analyses of pole cell migration in *Drosophila*

The relatively simple genetics available in *Drosophila* has lead to the discovery of several controls of pole cell migration. Genes that are expressed either in the somatic cells or in the pole cells have been shown to affect migration (For a review, see Santos, 2004a). Migration begins as the pole cells are carried into the embryo along the PMG epithelium, and subsequently migrate through this epithelium. Here, mutations in *trapped-in-endoderm1* (*tre-1*), a G-protein coupled receptor expressed in the pole cells, affect the migration of pole cells across the PMG epithelium (Kunwar, 2003). The pole cells of *tre1* mutant embryos are unable to exit the PMG, presumably because they are unable to transduce a signal to move across this epithelium. Consistent with this interpretation, pole cells of *tre1* mutant embryos which escape early from the midgut are capable of migrating properly to the gonads, indicating that mutations in *tre1* do not affect motility overall. A similar phenotype to that of *tre-1* occurs in *serpent* (*srp*) and

huckebein (*hkb*) mutants (Jaglarz and Howard, 1995, Moore et al., 1998). These mutations affect the dissolution of apical junctions between the cells in the PMG. These gaps have been shown to be required for the passage of the pole cells through the PMG by ultrastructural studies (Callaini, 1995, Jaglarz and Howard, 1995). Mutations in the Jak/Stat signaling pathway also affect early pole cell migration. Li et al. (2003) demonstrated that constitutive activation of Torso signaling overactivates Stat in the pole cells of early embryos, leading to an overproliferation of pole cells which exit the midgut early and migrate errantly through the mesoderm. Loss-of-function mutations in *tor* or in *stat* render the pole cells immotile, and the pole cells remain outside the embryo, even at later stages.

Once wildtype pole cells exit the PMG, they will migrate dorsally and separate into two groups that will eventually associate with the somatic cells of the gonad. Wunen and Wunen2 are lipid phosphate phosphatases, which are required in both the pole cells and the soma. They control the migration and survival of the pole cells as they migrate towards the mesoderm (Hanyu-Nakamura, 2004, Renault et al., 2004, Sano, 2005, Starz-Gaiano et al., 2001, Zhang et al., 1997, Zhang, 1996). In the soma, the Wunens are expressed in the ventral portion of the PMG and in the central nervous system, to repel the pole cells dorsally and aid in the bifurcation of the pole cells. In the pole cells, Wunen and Wunen-2 affect the survival of the pole cells, so that any pole cells left in the middle of the embryo after exit from the midgut are eliminated. In addition, the germ plasm components, *nos, pumillo (pum)* and *pgc*, are required at this step, since the pole cells of embryos from mutant mothers cluster together and do not migrate dorsally into the mesoderm (Asaoka-Taguchi, 1999, Forbes and Lehmann, 1999, Kobayashi et al., 1996, Nakamura et al., 1996). Association of the pole cells with the mesoderm is also disrupted in embryos mutant for *zinc finger homeodomain protein-1* (*zfh-1*), *tinman* (*tin*) and

brachyenteron (*byn*) (Broihier et al., 1998, Moore et al., 1998). These mutants all affect specification of the mesoderm, leaving the germ cells to remain in the PMG.

Pole cell migration ends as the pole cells arrive at the SGPs. The fact that pole cells in *zfh-1, tin,* and *byn* mutants remain in the PMG suggested that the mesoderm might provide an attractant for the germ cells. Van Doren et al. (1998) showed that ectopic expression of a key enzyme of the cholesterol/isoprenoid pathway, 3-Hydroxy 3-Methylglutaryl Coenzyme A Reductase (HMGCoAR or *Hmgcr* (*Columbus, clb*), is sufficient to attract the pole cells to the source of expression. In addition, *clb* is normally expressed in the mesoderm, and its expression is restricted to the SGPs as embryos mature. The enzymes Farnesyl-diphosphate synthase (encoded by *fpps*), Geranylgeranyl diphosphate synthase (*quemao*) and Geranylgeranyl transferase type 1 (*βGGT1*) act downstream of HMGCoAR, indicating that it is the isoprenoid biosynthesis pathway which may act to produce a germ cell attractant, although the nature of the attractant is still unknown (Santos, 2004b).

OVERVIEW OF THE DISSERTATION RESEARCH

There are two main aims of the research in this thesis. The first is to determine if the novel protein Oskar has any further role in polar granule formation beyond their initial nucleation, and the second is to determine the function of *Bluestreak* in pole cell formation and migration.

To investigate a further role for Oskar in polar granule formation, I cloned the *oskar* gene from *D. immigrans* flies (osk^{imm}) and introduced it into *D. melanogaster* flies using P-element transformation. I found that osk^{imm} was able to rescue both the posterior patterning and pole cell formation defects of embryos from *oskar* mutant mothers (Lehmann and Nüsslein-Volhard, 1986). In addition, I found that the polar granules of

embryos containing only Osk^{imm} as a source of Oskar protein are oblong in shape by gastrulation, vastly different from the individual, spherical granules of wildtype *D. melanogaster* embryos. The pole cells formed solely by the action of the Osk^{imm} protein are fully functional, as they are competent to populate the gonad and produce ovaries in adult daughters. Interestingly, when *osk^{imm}* is introduced into otherwise wildtype flies, the polar granules resemble those of *D. immigrans* embryos, indicating that the Osk^{imm} protein is able to dominantly alter the shape of the polar granules.

I have also identified a novel protein important for both pole cell formation and migration, Bluestreak. Initially, I found that embryos from mothers heterozygous for deficiencies deleting *Bluestreak* showed defects in pole cell number and shape. I subsequently made P-element excision mutants in *Bluestreak* and found that the pole cell defects are indeed due to a reduction in Bluestreak and these mutations are both dominant and maternal. Ovaries of *Blue^{-}* females have defects in the localization of Staufen and Oskar, sufficient to cause a reduction in pole cell number in embryos (Webster et al., 1994). I also found that mutations in *fpps, clb,* and *qm* dominantly suppress the defects in pole cell shape, implicating Bluestreak in the process of pole cell migration. Finally, I found that Bluestreak localizes to centrosomes along with γ-tubulin throughout the embryo, and to the nuclear membrane in pole cells. My findings introduce the possibility that Bluestreak may act to regulate germ cell migration in *Drosophila*.

REFERENCES

Aravin, A. A., Klenov, M., Vagin, V.V., Bantignies, F., Cavalli, G., Gvozdev, V.A. 2004. Dissection of a natural RNA silencing process in the *Drosophila melanogaster* germ line. *Mol Cell Biol.* 24:6742-6750.

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K., Kobayashi, S. 1999. Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. *Nat Cell Biol.* 1:431-437.

Bardsley, A., K. McDonald, and R. E. Boswell. 1993. Distribution of tudor protein in the *Drosophila* embryo suggests separation of functions based on site of localization. *Development.* 119:207-219.

Boswell, R. E., and A. P. Mahowald. 1985. *tudor*, a gene required for assembly of the germ plasm in Drosophila melanogaster. *Cell.* 43:97-104.

Breitwieser, W., F.-H. Markussen, H. Horstmann, and A. Ephrussi. 1996. Oskar protein interaction with vasa represents an essential step in polar granule assembly. *Genes Dev.* 10:2179-2188.

Broihier, H. T., L. A. Moore, M. Van Doren, S. Newman, and R. Lehmann. 1998. zfh-1 is required for germ cell migration and gonadal mesoderm development in Drosophila. *Development.* 125:655-666.

Callaini, G., Riparbelli, M.G., Dallai, R. 1995. Pole cell migration through the gut wall of the Drosophila embryo: analysis of cell interactions. *Dev Biol.* 170:365-375.

Campos-Ortega, J. A. a. H., Volker. 1985. The Embryonic Development of Drosophila melanogaster.

Cerutti, L., N. Mian, and A. Bateman. 2000. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the piwi domain. *Trends Biochem Sci.* 25:481-482.

Chiquione, A. D. 1954. The identification, origin and migration of the primordial germ cells of the mouse embryo. *Anat Rec.* 118:135-146.

Christerson, L. B., and D. M. McKearin. 1994. *orb* is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis. *Genes Dev.* 8:614-628.

Counce, S. J. 1963. Developmental morphology of polar granules in Drosophila including observations on pole cell behavior and distribution during embryogenesis. *J. Morph.* 112:129-145.

Deshpande, G., Calhoun, G., Schedl, P. 2004. Overlapping mechanisms function to establish transcriptional quiescence in the embryonic Drosophila germline. *Development.* 131:1247-1257.

Eddy, E. M. 1975. Germ plasm and the differentiation of the germ cell line. *Int. Rev. Cytol.* 43:229-280.

Eddy, E. M. 1996. Origin and migration of primordial germ cells in mammals. *Dev Genet.* 19:287-289.

Ephrussi, A., and R. Lehmann. 1992. Induction of germ cell formation by *oskar*. *Nature.* 358:387-392.

Erdélyl, M., A.-M. Michon, A. Guichet, J. B. Glotzer, and A. Ephrussi. 1995. Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. *Nature.* 377:524-527.

Extavour, C. G., and M. Akam. 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development.* 130:5869-5884.

Forbes, A., and R. Lehmann. 1999. Cell migration in Drosophila. *Curr Opin Genet Dev.* 9:473-478.

Gavis, E. R., and R. Lehmann. 1994. Translational regulation of *nanos* by RNA localization. *Nature.* 369:315-318.

Gavis, E. R., L. Lunsford, S. E. Bergsten, and R. Lehmann. 1996. A conserved 90 nucleotide element mediates translational repression of *nanos* RNA. *Development.* 122:2791-2800.

Geigy, R. 1931. Action de l'ultra-violet sur le pole germinale dans l'oeuf de Drosophila melanogaster. *Rev. Suisse Zool.* 38:187-288.

Godin, I., Wylie, C., Heasman, J. 1990. Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture. *Development.* 108:357-363.

Gomperts, M., Wylie, C., Heasman, J. 1994. Primordial germ cell migration. *Ciba Foundation Symposium.* 182:121-139.

Hanyu-Nakamura, K., Kobayashi, S., Nakamura, A. 2004. Germ-cell autonomous Wunen2 is required for germline development in Drosophila embryos. *Development.* 131:4545-4553.

Harris, A. N., and P. M. Macdonald. 2001. Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development.* 128:2823-2832.

Hay, B., L. Ackerman, S. Barbel, L. Jan, and Y. N. Jan. 1988. Identification of a component of *Drosophila* polar granules. *Development.* 103:625-640.

Hegner, R. W. 1914. The Germ-Cell Cycle in Animals.

Illmensee, K., and A. P. Mahowald. 1974. Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. *Proc. Natl. Acad. Sci. USA.* 71:1016-1020.

Illmensee, K., and A. P. Mahowald. 1976. The autonomous function of germ plasm in a somatic region of the *Drosophila* egg. *Exp. Cell Res.* 97:127-140.

Jaglarz, M. K., Howard, K.R. 1994. Primordial germ cell migration in Drosophila melanogaster is controlled by somatic tissue. *Development.* 120:83-89.

Jaglarz, M. K., and K. R. Howard. 1995. The active migration of *Drosophila* primordial germ cells. *Development.* 121:3495-3503.

Jongens, T. A., L. D. Ackerman, J. R. Swedlow, L. Y. Jan, and Y. N. Jan. 1994. *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Dev.* 8:2123-2136.

Kashikawa, M., R. Amikura, and S. Kobayashi. 2001. Mitochondrial small ribosomal RNA is a component of germinal granules in Xenopus embryos. *Mech Dev.* 101:71-77.

Kim-Ha, J., J. L. Smith, and P. M. Macdonald. 1991. *oskar* mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell.* 66:23-35.

Kim-Ha, J., K. Kerr, and P. M. Macdonald. 1995. Translational regulation of *oskar* mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell.* 81:403-412.

Kim-Ha, J., P. J. Webster, J. L. Smith, and P. M. Macdonald. 1993. Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development.* 119:169-178.

Kim, J., Daniel, J., Espejo, A., Lake, A., Krishna, M., Xia, L., Zhang, Y., Bedford, M.T. 2006. Tudor, MBT and chromo domains guage the degree of lysine methylation. *EMBO J.* 7:397-403.

King, R. C. 1970. Ovarian development in *Drosophila melanogaste*r.

Knaut, H., F. Pelegri, K. Bohmann, H. Schwarz, and C. Nusslein-Volhard. 2000. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. *J Cell Biol.* 149:875-888.

Kobayashi, S., and M. Okada. 1989. Restoration of pole-cell-forming ability to u.v.-irradiated *Drosophila* embryos by injection of mitochondrial lrRNA. *Development.* 107:733-742.

Kobayashi, S., M. Yamada, M. Asaoka, and T. Kitamura. 1996. Essential role of the posterior morphogen nanos for germline development in *Drosophila*. *Nature.* 380:708-711.

Komiya, T. a. T., Y. 1995. Cloning of a gene of the DEAD box protein family which is specifically expressed in germ cells in rats. *Biochem Biophys Res Commun.* 207:405-410.

Kunwar, P. S., Starz-Gaiano, M., Bainton, R.J., Heberlein, U., Lehmann, R. 2003. Tre1, a G-protein-coupled receptor, directs transepithelial migration of Drosophila germ cells. *PloS Biol.* 3:E80.

Lantz, V., J. S. Chang, J. I. Horabin, D. Bopp, and P. Schedl. 1994. The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* 8:598-613.

Lasko, P. F., and M. Ashburner. 1988. The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. *Nature.* 335:611-617.

Lasko, P. F., and M. Ashburner. 1990. Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. *Genes Dev.* 4:905-921.

Leatherman, J. L., L. Levin, J. Boero, and T. A. Jongens. 2002. germ cell-less acts to repress transcription during the establishment of the Drosophila germ cell lineage. *Curr Biol.* 12:1681-1685.

Lehmann, R., and C. Nüsslein-Volhard. 1986. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in Drosophila. *Cell.* 47:141-152.

Li, J., Xia, F., Li, W.X. 2003. Coactivation of STAT and Ras is required for germ cell proliferation and invasive migration in Drosophila. *Dev Cell.* 5:787-798.

Liang, L., W. Diehl-Jones, and P. Lasko. 1994. Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. *Development.* 120:1201-1211.

Lin, H. 2007. piRNAs in the germ line. *Science.* 316:397.

Macdonald, P. M., and G. Struhl. 1988. *Cis*-acting sequences responsible for anterior localization of *bicoid* mRNA in *Drosophila* embryos. *Nature.* 336:595-598.

Mahowald, A. P. 2001. Assembly of the Drosophila germ plasm. *Int Rev Cytol.* 203:187-213.

Mahowald, A. P., K. Illmensee, and F. R. Turner. 1976. Interspecific transplantation of polar plasm between Drosophila embryos. *J Cell Biol.* 70:358-373.

Mahowald, A. P. 1962. Fine structure of pole cells and polar granules in *Drosophila melanogaster*. *J. exp. Zool.* 151:201-216.

Mahowald, A. P. 1968. Polar granules of *Drosophila*. II. Ultrastructural changes during early embryogenesis. *J. exp. Zool.* 167:237-262.

Mahowald, A. P. 1971a. Polar granules of *Drosophila*. III. The continuity of polar granules during the life cycle of *Drosophila*. *J. exp. Zool.* 176:329-344.

Mahowald, A. P. 1971b. Polar granules of *Drosophila*. IV. Cytochemical studies showing loss of RNA from polar granules during early embryogenesis. *J. exp. Zool.* 176:345-352.

Martinho, R. G., P. S. Kunwar, J. Casanova, and R. Lehmann. 2004. A noncoding RNA is required for the repression of RNApolII-dependent transcription in primordial germ cells. *Curr Biol.* 14:159-165.

Maurer-Stroh, S., Dickens, N.J., Hughes-Davies, L., Kousarides, T., Eisenhaber, F., Ponting, C.P. 2003. The Tudor domain 'Royal Family': Tudor, plant Agenet, Chromo, PWWP and MBT domains. *Trends Biochem Sci.* 28:69-74.

Megosh, H. B., D. N. Cox, C. Campbell, and H. Lin. 2006. The Role of PIWI and the miRNA Machinery in Drosophila Germline Determination. *Curr. Biol.* In press

Moore, L. A., H. T. Broihier, M. Van Doren, L. B. Lunsford, and R. Lehmann. 1998. Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila. *Development.* 125:667-678.

Murata, Y., and R. P. Wharton. 1995. Binding of pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. *Cell.* 80:747-756.

Nakamura, A., R. Amikura, M. Mukai, S. Kobayashi, and P. F. Lasko. 1996. Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. *Science.* 274:2075-2079.

Newmark, P. A., and R. E. Boswell. 1994. The *mago nashi* locus encodes an essential product required for germ plasm assembly in *Drosophila*. *Development.* 120:1303-1313.

Nieuwkoop, P. D. 1979. Primordial germ cells in the chordates: embryogenesis and phylogenesis.

Poulson, D. F. 1950. Histogenesis, organogenesis and differentiation in the embryo of Drosophila melanogaster. *In: Biology of Drosophila.* 168-274.

Raff, J. W., Glover, D.M. 1989. Centrosomes, not nuclei, initiate pole cell formation in Drosophila embryos. *Cell.* 57:611-619.

Raz, E. 2000. The function and regulation of vasa-like genes in germ-cell development. *Genome Biol.* 1:REVIEWS1017.

Renault, A. D., Y. J. Sigal, A. J. Morris, and R. Lehmann. 2004. Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science.* 305:1963-1966.

Renault, A. D., Lehmann, R. 2006. Follow the fatty brick road: lipid signaling in cell migration. *Curr Opin Genet Dev.* 16:348-354.

Rhoads, R. E. 1993. Regulation of eukaryotic protein synthesis by initiation factors. *J. Biol. Chem.* 268:3017-3020.

Rongo, C., and R. Lehmann. 1996. Regulated synthesis, transport and assembly of the Drosophila germ plasm. *Trends Genet.* 12:102-109.

Sano, H., Renault, A.D., Lehmann, R. 2005. Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen2. *J Cell Biol.* 171:675-683.

Santos, A. C., Lehmann, R. 2004a. Germ cell specification and migration in Drosophila and beyond. *Curr Biol.* 14:R578-89.

Santos, A. C., Lehmann, R. 2004b. Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Dev Cell.* 6:283-293.

Schupbach, T., Weischaus, E. 1989. Female sterile mutations on the second chromosome of Drosophila melanogaster. I. Maternal effect mutations. *Genetics.* 121:101-117.

Seydoux, G. a. S., T. 2001. The germline in *C. elegans*: origins, proliferation and silencing. *Int Rev Cytol.* 203:139-185.

Sonnenblick, B. P. 1941. Germ cell movements and sex differentiation of the gonads in the Drosophila embryo. *Proc Natl Acad Sci U S A.* 27:484-489.

Sonnenblick, B. P. 1950. The early embryology of Drosophila melanogaster. *In: Biology of Drosophila.* 168-274.

St Johnston, D. 1993. Pole plasm and the posterior group genes. *In* The Development of *Drosophila melanogaster(1)*, Bate, M., and A. M. Arias, editors. Cold Spring Harbor Laboratory Press, New York. 325-363.

Starz-Gaiano, M., N. K. Cho, A. Forbes, and R. Lehmann. 2001. Spatially restricted activity of a Drosophila lipid phosphatase guides migrating germ cells. *Development.* 128:983-991.

Starz-Gaiano, M., and R. Lehmann. 2001. Moving towards the next generation. *Mech Dev.* 105:5-18.

Technau, G. M., and J. A. Campos-Ortega. 1986. Lineage analysis of transplanted individual cells in embryos of *Drosophila melanogaster* III. Commitment and proliferative capabilities of pole cells and midgut progenitors. *Roux's Arch. Dev. Biol.* 195:489-498.

Thomson, T., and P. Lasko. 2004. Drosophila tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. *Genesis.* 40:164-170.

Underwood, E. M., J. H. Caulton, C. D. Allis, and A. P. Mahowald. 1980. Developmental fate of pole cells in Drosophila melanogaster. *Dev Biol.* 77:303-314.

Van Doren, M., A. L. Williamson, and R. Lehmann. 1998. Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol.* 8:243-246.

Waring, G. L., C. D. Allis, and A. P. Mahowald. 1978. Isolation of polar granules and the identification of polar granule-specific protein. *Dev. Biol.* 66:197-206.

Webster, P. J., J. Suen, and P. M. Macdonald. 1994. *Drosophila virilis oskar* transgenes direct body patterning but not pole cell formation or maintenance of mRNA localization in *D. melanogaster*. *Development.* 120:2027-2037.

Wilson, E. B. 1896. The cell in development and inheritance.

Wilson, J. E., J. E. Connell, J. D. Schlenker, and P. M. Macdonald. 1996. Novel genetic screen for genes involved in posterior body patterning in Drosophila. *Dev Genet.* 19:199-209.

Zalokar, M. a. E., I. 1976. Autoradiographic studies of protein and RNA forming during early development of *Drosophila* eggs. *Dev Biol.* 49:425-437.

Zhang, N., J. Zhang, K. J. Purcell, Y. Cheng, and K. Howard. 1997. The Drosophila protein Wunen repels migrating germ cells. *Nature.* 385:64-67.

Zhang, N., Zhang, J., Cheng, Y., Howard, K. 1996. Identification and genetic analysis of wunen, a gene guiding Drosophila melanogaster germ cell migration. *Genetics.* 143:1231-1241.

Chapter 2: Oskar controls the morphology of polar granules and nuclear bodies in *Drosophila*

Jennifer R. Jones and Paul M. Macdonald

Institute for Cellular and Molecular Biology

Section of Molecular Cell and Developmental Biology

The University of Texas at Austin

Austin, TX 78712-0159

Running title: Oskar controls polar granule morphology

Key words: *Drosophila*, Oskar, pole cell, polar granules, nuclear bodies

ABSTRACT

Germ cell formation in *Drosophila* relies on polar granules, large ribonucleoprotein complexes found at the posterior end of the embryo. The granules undergo characteristic changes in morphology during development, including the assembly of multiple spherical bodies from smaller precursors. Several polar granule components, both protein and RNA, have been identified. One of these, Oskar protein, acts to initiate granule formation during oogenesis and to recruit other granule components. To ask if Oskar has a continuing role in the organization of the granules and control of their morphology, we took advantage of species-specific differences in polar granule structure. The polar granules of *D. immigrans* fuse into a single large oblong aggregate, as opposed to the multiple distinct spherical granules of *D. melanogaster* embryos. We cloned the *D. immigrans oskar* gene and expressed it in *D. melanogaster* embryos. *D. immigrans oskar* not only rescues the body patterning and pole cell defects of embryos from *D. melanogaster oskar*$^{-}$ mothers, but also converts the morphology of the polar granules to that of *D. immigrans*. The nuclear bodies, structures which appear to be closely related to polar granules, are also converted to the *D. immigrans* type morphology. We conclude that *oskar* plays a persistent role in the polar granules, first initiating their formation and later controlling their organization and morphology.

INTRODUCTION

Pole plasm is a specialized cytoplasm found at the posterior pole of *Drosophila* oocytes. Electron-dense structures known as polar granules form within this cytoplasm by stage 10 of oogenesis and are incorporated into the pole cells (germ cells) by stage 3 of

embryogenesis. Classical experiments have demonstrated the necessity of the pole plasm for forming germ cells. Geigy (1931) showed that UV irradiation of the pole plasm of embryos produced sterile adult flies. Illmensee and Mahowald (1974) extended this by showing that pole plasm was sufficient to form pole cells independent of its normal subcellular distribution. Transplantation experiments were performed in which the pole plasm was removed from the posterior of donor embryos and inserted into the anterior pole of recipients. This transplanted cytoplasm formed ectopic pole cells, which upon transplantation to their normal position at posterior pole of another recipient embryo formed functional germ cells.

Stronger evidence that the pole cell forming activity was contained within the polar granules, rather than posterior cytoplasm, was obtained once polar granule components were identified. The isolation of maternal effect mutants defective in embryonic body patterning revealed one group – the *grandchildless-knirps* genes – that have posterior patterning defects and fail to form pole cells (Boswell and Mahowald, 1985, Lehmann and Nüsslein-Volhard, 1986, Schüpbach and Wieschaus, 1986). At least four members of this group, *aubergine*, *oskar*, *tudor* and *vasa*, encode proteins that are concentrated in polar granules (Bardsley et al., 1993, Breitwieser et al., 1996, Harris and Macdonald, 2001, Hay et al., 1988, Lasko and Ashburner, 1990). Notably, mislocalization of Osk protein to the anterior, using a transgene containing the *osk* coding region and *bicoid* anterior mRNA localization signal, induces the formation of anterior polar granules and pole cells. All of the known polar granule components are recruited to the site of ectopic polar granules, and the pole cells that form are functional germ cells (Ephrussi and Lehmann, 1992, Megosh et al., 2006, Nakamura et al., 1996). Given the strong correlation between the presence of polar granules and the formation of germ cells,

both at the natural site and ectopically, it is generally accepted that polar granules specify germ cell formation (Saffman and Lasko, 1999).

The behavior of the mislocalized Osk protein clearly demonstrates that Osk can nucleate polar granule formation. This property appears unlikely to be shared by other polar granule components, as there have been no reports that anterior localization of mRNAs encoding other polar granule components can induce ectopic granule formation (Megosh et al., 2006). This special property of Osk may be related to the fact that it becomes anchored to the oocyte cortex and is not free to diffuse. Osk protein is synthesized from *osk* mRNA localized at the posterior of the oocyte, and because of anchoring remains concentrated there (Kim-Ha et al., 1991, Rongo et al., 1995, Vanzo and Ephrussi, 2002, Webster et al., 1994). Tight anchoring requires the long form of Osk protein, which includes an amino terminal extension not present in short Osk (Markussen et al., 1995, Vanzo and Ephrussi, 2002). Variants of Osk unable to anchor do not support pole cell formation, although they can be very active in posterior body patterning activity (Vanzo and Ephrussi, 2002, Webster et al., 1994). Thus it appears that diffuse Osk can direct posterior body patterning, while specification of germ cells requires a more concentrated zone of Osk. The anchoring may thus serve to maintain a local concentration at a level sufficient to efficiently bind and recruit other polar granule components. This raises a question about the role of Osk in polar granule formation: does it serve only to recruit other factors, or does it have a more active or persistent role in the structure and organization of polar granules?

Our understanding of polar granule organization stems primarily from ultrastructural studies (Mahowald et al., 1976, Mahowald, 1962, Mahowald, 1968). The granules first appear at the posterior end of *D. melanogaster* oocytes as an abundance of small particles, which later fuse to form larger, spherical structures with electron-lucid

cores. By gastrulation, each pole cell contains a few dispersed polar granules, which persist until the pole cells migrate through the midgut primordium. In parallel, nuclear bodies, with the same spherical structure, form in nuclei (Harris and Macdonald, 2001, Mahowald et al., 1976). During pole cell migration, the polar granules begin to fragment and associate with the outer nuclear envelope, resembling nuage (termed fibrous bodies by Mahowald, 1971).

Despite knowledge of the ultrastructural features of the granules and the identification of several components, our understanding of the relative organization of the components within the granules is still lacking. Oskar and Vasa interact by two-hybrid analysis (Breitwieser et al., 1996), however it remains unclear how these two proteins associate with each other in the granules. There have been no reports of crystallographic studies of the granules to determine their structure at the molecular level, since approaches such as these would require pure granules, which have so far been elusive to obtain.

Counce (1963) and Mahowald (1968) have studied the ontogeny of the granules in several *Drosophila* species. Both found that the granules display species-specific differences in fusion and configuration of the granules within the cell, beginning with pole cell formation and into gastrulation. Notably, in *Drosophila immigran*s, the polar granules fuse into one large sheet of polar granule material (Mahowald et al., 1976, Mahowald, 1968), as opposed to the multiple, spherical, individual granules that are characteristic of *Drosophila melanogaster*. If Osk has a persistent role and can dictate polar granule morphology, then introduction of the *Drosophila immigrans osk* gene into *D. melanogaster* might convert the granules into *D. immigrans* type. This provides an opportunity to ask if Osk has an organizational role beyond the initial recruitment of other components.

Here we show that Osk is persistently present in polar granules from their formation to their fragmentation, and is also found in the closely related nuclear bodies that display the same spherical shape. Expression in *D. melanogaster* of the *osk* gene from *D. immigrans* disrupts the spherical organization of polar granules and converts them to the *D. immigrans* shape, and also shifts the morphology of the nuclear bodies towards that of the *D. immigrans* type. Our results show that Osk plays a continuing role in polar granule organization, and may be the primary determinant of the underlying arrangement of both of these structures.

RESULTS

Persistence of Osk protein in pole cells during embryogenesis

Osk protein is localized to the posterior pole of oocytes from the onset of its accumulation, and is intimately associated with polar granules as they form (Breitwieser et al., 1996, Kim-Ha et al., 1995, Markussen et al., 1995, Rongo et al., 1995, Wilson et al., 1996). Although Osk protein continues to be present in polar granules in early embryos, at least up to the initiation of gastrulation (Harris and Macdonald, 2001), the duration of this association has not been examined. To address this issue, we monitored Osk by immunofluorescence in embryos up to the point when pole cells arrive at and populate the gonad. The distribution of Osk in embryos up to blastoderm stage has been described (Harris and Macdonald, 2001), we repeat it here to provide the basis for comparison to other genotypes analyzed in this report. Before pole buds form, Oskar appears in a crescent at the posterior end of the embryo, in small sand-like granules, approximately 0.2 - 0.3 μm in diameter (Fig. 2.1A). Starting at the cellular blastoderm stage (Fig. 2.1D), the small granules begin to coalesce to form spherical granules (0.7 –

1.4 μm in diameter), structures that were first visualized by electron microscopy and are sufficiently large to be readily recognized by light microscopy. Much like the polar granule marker GFP-Aub (Fig. 2.1D'), these spheres appear in confocal cross sections as donut shapes when stained for Osk.

Ultrastructural studies have shown that the spherical granules persist through gastrulation, but begin to fragment as the pole cells migrate through the midgut primordium (Mahowald, 1971). At this point the granules adopt a granular perinuclear distribution that characterizes nuage, a structure found in the germ line cells of many or all animals (Saffman and Lasko, 1999). Osk remains persistently associated with polar granules for the duration of their life as spherical particles, and Osk continues to display the donut appearance. Once the spheres fragment Osk becomes essentially undetectable (Fig. 2.1G, H). Some Osk may still be present, but difficult to detect once the protein is dispersed as the spherical structures break down. However, it is quite clear that Osk is a perduring component of polar granules for their entire life as spherical structures, from cellular blastoderm stage to the migration of the pole cells through the midgut primordium.

Polar granules are cytoplasmic, but bodies with similar structure appear in pole cell nuclei at stage 4 of embryogenesis (syncytial blastoderm). So far, only Oskar and Vasa have been shown to be components of the nuclear bodies (Harris and Macdonald, 2001). GFP-Aub is not detected in nuclei (Fig. 2.1I'), facilitating the identification of nuclear regions. Osk is first detected in pole cell nuclei at the syncytial blastoderm stage (Fig. 2.1D'). The Osk-containing structures (0.3 - 0.5 μm in diameter) also appear as "donuts" by confocal microscopy, and match the description previously noted for *D. melanogaster* nuclear bodies (Mahowald et al., 1976). Just as described for nuclear bodies, the Osk-containing structures coalesce and grow larger by gastrulation (0.4 - 0.7 μm in diameter,

Fig 2.1I, I''), and also become harder to detect as the pole cells begin to migrate. Given the striking similarities in nuclear bodies and the Osk-containing structures, we conclude that Osk is in nuclear bodies. The function of the nuclear bodies is unknown, and so the significance of the Osk in these bodies, in terms of pole cell function, is uncertain. However, the presence of Osk in the nuclear bodies is consistent with a role for Osk in organizing the structure of the spheres.

Osk^{imm} is active and functional in *D. melanogaster*

The polar granules of *D. immigrans* embryos form one large sheet of granule material per cell, and are distinctively different from the spherical, individual granules of *D. melanogaster*. If Osk plays a role in organizing the structure of polar granules, then expression of *D. immigrans osk* in *D. melanogaster* might transform the polar granules from individual spheres to one or a few aggregates per cell.

To obtain the *D. immigrans osk* gene, a bacteriophage lambda library of genomic *D. immigrans* DNA was constructed and screened by low stringency hybridization with a *D. melanogaster osk* probe. Several overlapping clones were recovered, and the region corresponding to the *osk* homolog was sequenced; for simplicity we refer to this gene as *Osk^{imm}* (Genbank accession number: DQ823083), and the *D. melanogaster* gene as *osk*.

Two transgenes were used to express Osk^{imm} in *D. melanogaster* (Fig. 2.2A). One, *P[osk^{imm}]*, provides a large genomic DNA fragment containing the *osk^{imm}* coding region and 5 kb of 5' and 2 kb of 3' flanking sequences. Genomic DNA transgenes of *D. melanogaster osk* with smaller flanking regions are strongly expressed and fully rescue *osk^{-}* flies, suggesting that the *P[osk^{imm}]* transgene will include all transcriptional control elements.

The *osk* gene is subject to extensive post-transcriptional control, relying on regulatory elements contained within the mRNA. Most of these elements lie within the 3' UTR (Gunkel et al., 1998, Kim-Ha et al., 1993) (Kim-Ha et al., 1995) (Munro et al., 2006). In an effort to ensure that the osk^{imm} gene would be regulated properly in *D. melanogaster*, a second transgene was also tested. *P[*$Osk^{imm3'mel}$*]* is identical to *P[*Osk^{imm}*]* except that the region corresponding to the osk^{imm} 3' UTR and 3' flanking sequences was replaced with the equivalent *D. melanogaster* sequences.

Several independent transgenic lines were established for each transgene, as well as a *D. melanogaster* genomic *osk* transgene, *P[*osk^{+}*]*. Representative examples were introduced into the osk^{54}*/Df(3R)pXT103* background (noted for the remainder of the text as osk^{54}*/Df*), which is null for Osk protein. The phenotypes below are dependent only on the maternal genotype, so for simplicity, the embryos will be described by their mother's genotype, not their own; this convention is also used in the figure legends.

The control osk^{54}*/Df* embryos fail to form polar granules and pole cells and lack all abdominal denticle belts, reflecting the two activities of Osk in germ cell formation and posterior body patterning (Lehmann and Nüsslein-Volhard, 1986). One copy of *P[*osk^{+}*]* completely rescues the patterning phenotype (Fig. 2.2B). At a low frequency, the *P[*osk^{+}*]; *osk^{54}*/Df* embryos display minor head defects, a phenotype commonly due to excessive or ectopic expression of Osk. Pole cell formation is sensitive to the level of *osk* activity (Lehmann and Nüsslein-Volhard, 1986, Smith et al., 1992), and the single copy of the transgene is less effective than two copies of the endogenous *osk* for this activity: *P[*osk^{+}*]; *osk^{54}*/Df* embryos average 23.6 pole cells per embryo at nuclear cycle 14, as compared to 36.2 pole cells in wild type embryos of the same stage (Fig. 2.2C).

Both Osk^{imm} transgenes provide substantial body patterning activity, as abdominal segmentation is fully restored (Fig 2.2B). In addition, the frequency of head defects is

consistently elevated (as compared to *osk*$^{-}$ embryos expressing the *P[osk*$^{+}$*]* transgene). One of the *P[Osk*$^{imm3'mel}$*]* transgenic lines produces a large fraction of bicaudal embryos, but it has higher mRNA levels than the second line, or than either of the two lines of *P[osk*^{imm}*]* transgenics (Fig. 2.3H). The *osk*^{imm} transgenes also rescue germ cell formation, although less efficiently than *P[osk*$^{+}$*]* (Fig. 2.2C). Whereas in *P[osk*$^{+}$*]/+; osk*^{54}*/Df* embryos there are an average of 23.6 pole cells at nuclear cycle 14, there are only 7.6 pole cells per *P[Osk*^{imm}*]/+; osk*^{54}*/Df* embryo, and 10.7 pole cells per *P[Osk*$^{imm\ 3'mel}$*]/+; osk*^{54}*/Df* embryo.

Thus the *osk*^{imm} transgenes appear to have elevated body patterning activity, but reduced pole cell forming activity. A simple explanation of this phenomenon is that the *osk* activity from the transgenes may not be as tightly restricted to the posterior pole as is endogenous *osk* activity. Normally, Osk protein is efficiently anchored at the posterior cortex of the oocyte, where the protein first begins to accumulate, and remains highly concentrated at the extreme posterior of the early embryo. This anchoring involves an interplay between Osk protein and *osk* mRNA: the initial posterior localization of the mRNA ensures that Osk will be synthesized very close to the posterior cortex, and Osk protein in turn acts to anchor the mRNA at that site and maintain its posterior localization. The high local concentration of Osk that is achieved by this process appears to be important for the ability to form pole cells, but diffuse and less concentrated Osk is still competent to drive posterior patterning. If the anchoring process is impaired in the embryos expressing Osk^{imm}, then the observed phenotypes would be expected. This is precisely what was found previously, but to a greater extent, when the *D. virilis osk* gene (*osk*^{vir}) was expressed in *D. melanogaster* (Webster et al., 1994).

Examination of the distribution of *osk*^{imm} transgenic mRNAs indicates that they are indeed less tightly localized than is endogenous *osk* mRNA in a wild type embryo (Fig.

2.3). Both *osk*^{imm} and *osk*$^{imm\ 3'mel}$ messages are localized to the posterior, but not as efficiently localized as *osk* mRNA. The endogenous *osk* mRNA in the *osk*^{imm} transgenic embryos is only poorly localized. Since its localization is entirely dependent on Osk^{imm} protein, this indicates that Osk^{imm} is less effective at anchoring the *D. melanogaster osk* mRNA than the *osk*^{imm} mRNA.

Pole cells formed by Osk^{imm} are functional

The ability of Osk^{imm} to direct pole cell formation in *D. melanogaster* suggests but does not prove that these pole cells are functional, and can populate the gonads with germ cells. To address this issue the embryos from *P[Osk*^{imm}*]/+; osk*^{54}*/Df* and *P[osk*$^{imm3'mel}$*]/+; osk*^{54}*/Df* mothers were raised to adults and scored for the presence of ovaries and for the ability to produce offspring. All of the control females, those from *osk* mutant mothers with a *P[osk*$^{+}$*]* transgene, had two ovaries as in wild type. All of the females whose *osk*$^{-}$ mothers had a *P[Osk*^{imm}*]* or a *P[osk*$^{imm3'mel}$*]* transgene had at least one ovary (most had two), and all of these adult females produced embryos. Thus Osk^{imm} directs formation of functional pole cells.

Polar granules formed by Osk^{imm} have the *D. immigrans* morphology

The ability of Osk^{imm} to support the formation of functional pole cells which will populate the gonad, albeit at a lower level than Osk, demonstrates that functional polar granules must be formed, and allows us to ask if the granules have the morphology of *D. melanogaster* or have been transformed into the morphology of *D. immigrans*. GFP-Aub was used to visualize the polar granules. The *aub* gene is required for pole cell formation, and GFP-Aub co-localizes with Osk and Vas in polar granules (Harris and Macdonald, 2001); this work).

In early *P[Osk^{imm}]/+; osk^{54}/Df* embryos, polar granules are small and sand-like, between 0.2 - 0.4 μm in diameter (Fig. 2.4K). By cellular blastoderm (Fig. 2.4N), all of the smaller granules begin to accumulate in one area of the cell, so that by gastrulation (Fig. 2.4O), each pole cell contains a single large aggregate of granule material. This polar granule morphology persists until the pole cells begin to migrate. Expression of *P[osk$^{imm3'mel}$]* in *osk^{54}/Df* embryos has essentially the same effect (Fig. 2.4P-T). Thus Osk^{imm} confers the *D. immigrans* morphology on polar granules formed in a background where all components but Osk are of the *D. melanogaster* type. The control embryos, in which polar granule formation relies on Osk from the *P[osk^{+}]* transgene, have the typical appearance of *D. melanogaster* polar granules at all stages (Fig. 2.4F-J).

Osk^{imm} influences nuclear body morphology

Osk appears in polar granules in the pole cell cytoplasm and in nuclear bodies within the pole cell nuclei [(Harris and Macdonald, 2001); Fig 2.1,panel I]. The morphology of nuclear bodies also differs in the pole cells of *D. melanogaster* and *D. immigrans* embryos. In *D. melanogaster* the nuclear bodies first appear at syncytial blastoderm stage. They persist until the pole cells migrate to the gonad when they are no longer detectable. The nuclear bodies of *D. immigrans* are initially similar to those of *D. melanogaster*, displaying the characteristic donut appearance in cross section. However, during the cellular blastoderm stage they develop discontinuities, such that the spheres appear to be fragmented. Often, portions of multiple spheres aggregate (Mahowald et al., 1976). This difference allows us to ask if Osk^{imm} also controls nuclear body morphology. For experiments in which we examined the morphology of polar granules, GFP-Aub was used as a polar granule marker. The same approach cannot be used to

monitor the structure of nuclear bodies, as GFP-Aub does not enter the nucleus. The marker we have found to provide the strongest signal for nuclear bodies is Osk. We therefore tested the consequence of expressing Osk^{imm} in otherwise wild type flies, where the endogenous Osk is also present and can be used as a marker. Given the difference relative to the previous experiments - in which the only source of Osk is Osk^{imm} - we also examined polar granules to determine if Osk^{imm} could dominantly alter their morphology.

Polar granules were detected with both GFP-Aub and Osk antibodies, with effectively the same results. Just as when Osk^{imm} is the only Osk protein present, there is a dramatic shift from the *D. melanogaster* polar granule morphology to that of *D. immigrans* during cellular blastoderm and gastrulation stages (Fig. 2.5). For *P[osk^{imm}]* and *P[osk$^{imm3'mel}$]*, larger, oblong aggregates of polar granule material (between 1.5 – 3.4 μm long and 0.4 – 1.5 μm wide) begin to form in each pole cell. The aggregates are adjacent to the nucleus, and further coalesce, so that at gastrulation all of the granule material is found in one to three oblong structures per cell.

In the presence of Osk^{imm}, the nuclear bodies initially appear with the regular spherical morphology shared by both species up to the cellular blastoderm stage. At that point, *D. melanogaster* nuclear bodies maintain the same appearance, but *D. immigrans* nuclear bodies develop a fractured appearance and are no longer uniformly spherical. Similarly, the presence of Osk^{imm} in *D. melanogaster* pole cells causes the nuclear bodies to develop fissures, as seen in the donut cross sections, and the spheres become irregular and flattened (Fig. 2.5H and I). Because we are detecting the nuclear bodies with antibodies directed against Osk, the fissured appearance could reflect partitioning of Osk and Osk^{imm} within the bodies, such that Osk is specifically excluded from some regions, which then appear as gaps in the donuts. If this seemingly unlikely option is

correct, then the *D. immigrans*-like fracturing of the nuclear bodies could be an illusion. However, there is a second indication that the morphology of the nuclear bodies is altered: the bodies are transformed from regular spheres to the distorted spheroids that more closely resemble the *D. immigrans* nuclear bodies. We conclude that Osk^{imm} is a determinant for the morphology of both polar granules and nuclear bodies.

Structural differences between Osk and Osk^{imm}

The different properties of Osk and Osk^{imm} must be due to differences in their primary amino acid sequences. The Osk^{imm} sequence was aligned with that of Osk, as well as those of Osk proteins from a variety of other *Drosophila* species and one from *Anopheles gambiae* (Fig. 2.6A). The alignment reveals two regions of highest homology, separated by two regions that are less well conserved. The amino-terminal portion (region A in Fig. 2.6A) is the least well conserved with substantial differences in overall size and amino acid sequence. This region corresponds to the amino terminal extension that is present in only one of the two Osk isoforms. In *D. melanogaster,* the *osk* mRNA is translated from two different AUG codons, both in the same reading frame, to make long and short versions of Osk protein (Markussen et al., 1995). All of the *Drosophila osk* genes have the potential to make similar isoforms, with an invariant internal methionine codon at the appropriate position in each gene. Within the portion of Osk protein corresponding to the Short Osk isoform (which comprises the entire *A. gambiae osk* gene) there are two broad regions of high homology, one starting with the Short Osk methionine (region B, 69-92% identity) and the other at the carboxyl end of the protein (region D, 31-98% identity) and separated by a less well conserved region, C (26-83% identity)(note that the range in degree of homology for the less well conserved regions is very broad; this is due to the very close evolutionary distance between some of the

Drosophila species, and the corresponding high homology). The majority of molecularly characterized *osk* missense alleles are found in the most highly conserved D region (Kim-Ha et al., 1991) (Breitwieser et al., 1996).

Overall, the variation between Osk and the predicted Osk^{imm} protein sequences (Fig. 2.6B) is similar to that seen in comparison of other species of similar evolutionary divergence. Thus, the feature responsible for the striking difference in the polar granules of *D. melanogaster* and *D. immigrans* cannot be readily predicted.

Long and short isoforms of Osk^{imm} affect the polar granules differently

To determine whether the long or short isoform of Osk^{imm} contributes to the change in granule morphology, long and short Osk^{imm} transgenes were introduced into a wildtype background. The transgenes are identical to *P[Osk$^{imm3'mel}$]*, with the following changes. In *P[M1L-Osk$^{imm3'mel}$]* the first methionine codon is changed to encode leucine; this transgene will only make short Osk^{imm}. In *P[M103L-Osk$^{imm3'mel}$]* the methionine at position 103 is changed to a leucine, and in *P[M103,106L Osk$^{imm3'mel}$]* methionines at positions 103 and 106 are both altered to leucines. Although *D. melanogaster osk* has only a single methionine codon in this region of the protein, *D. immigrans* has the two methionine codons and either might serve to initiate translation of a short Osk^{imm} isoform. One or both of these transgenes should encode only the long isoform of Osk^{imm}.

The transgenes were introduced into the wildtype background to assess the morphology of the polar granules. All produce transcripts that are present at similar levels (data not shown). Embryos from mothers carrying the *P[M1L-Osk$^{imm3'mel}$]* transgene have granules that have been transformed to the *D. immigrans* type (Fig. 2.7B). Thus the short isoform of Osk^{imm} is sufficient to specify polar granule morphology.

The *P[M103L-Osk$^{imm3'mel}$]* and *P[M103,106L Osk$^{imm3'mel}$]* transgenes differ in their activities. When only the first of the two methionines near the predicted start of short Osk^{imm} is mutated, the transgene can still transform the polar granules to the *D. immigrans* type (Fig 2.7C). However, when both methionine codons that could initiate short Osk^{imm} are mutated, then the ability to alter polar granule morphology is lost (Fig 2.7D). The strong suggestion from these results is that either methionine can initiate translation of short Osk^{imm}, and that the long Osk^{imm} isoform does not contribute to specification of polar granule morphology.

DISCUSSION

Here we have shown that Oskar protein perdures in the granules during the time that the granules form and take on their characteristic "donut" shape in *Drosophila melanogaster*. We have also shown that the expression in *D. melanogaster* of *osk* from *D. immigrans* is sufficient to change the polar granule and nuclear body morphology of *D. melanogaster* embryos. From our data we conclude that Oskar plays a key role in determining the ultimate organization of both polar granules and nuclear bodies.

Investigation into the function of Oskar in polar granules has so far focused on the initiation of pole plasm assembly and recruitment by Oskar of other factors to this specialized cytoplasm. The role of Osk as the nucleator and a limiting factor for granule formation has been clearly demonstrated (Ephrussi and Lehmann, 1992, Smith et al., 1992) and these experiments have reinforced the idea that the presence of polar granules is sufficient for pole cell formation. While the initial formation of polar granules clearly relies on Osk, little is known about how the various components contribute to the

subsequent progression of granule assembly, transformation to spheres, and appearance of nuclear bodies. The Osk, Vas, Aub and Tud proteins are all involved, as mutants of each of the encoding genes either reduces or eliminates the granules. However, the functions of each individual component have so far not clarified how these components might work together to form the granules, or exactly what the granules do once formed. Vasa is a DEAD box helicase, which has been shown to bind and unwind RNA, and is homologous to eIF4A (Lasko and Ashburner, 1988, Hay et al., 1988,Liang et al., 1994). Aub is homologous to eIF2C, and contains PIWI and PAZ domains, which have been implicated in RNAi (Harris and Macdonald, 2001, Cerutti et al., 2000). Aub has been shown to be important for RNAi in the oocyte, as *aub* mutants lead to defects in RNAi activation and translational control. (Kennerdell et al., 2002) Aub is also required for the efficient translation of Osk and Grk in the ovary (Wilson et al., 1996). Several mutants in Tud result in smaller and fewer granules, raising the possibility of a role in fusion of granule material (Boswell and Mahowald, 1985, Thomson and Lasko, 2004). Recently, a detailed dissection of Tud revealed a critical role of Tud domain 1 (tud^{A36} mutant) in germ cell formation and polar granule formation, and led to the proposal that the Tud domains serve as docking platforms for polar granule assembly. Notably, mutation of a single Tud domain altered polar granule morphology from the small, sand-like spherical granules seen in wild type embryos at egg lay, to string-like granules (Arkov et al., 2006). Thus Osk, with its central role in specifying granule morphology, may perform this role via interactions with Tud. We compared, by yeast two hybrid assays, the interactions of the two Osk proteins with Tud, as well as the other known *D. melanogaster* polar granule components (Breitwieser et al., 1996), but found no substantial differences (data not shown).

To ask if Osk has a continuing role in polar granules, beyond the initial step in their formation, we extended the approach taken earlier by Mahowald et al. (1976). They found that transplantation of *D. immigrans* posterior polar plasm into *D. melanogaster* embryos resulted in polar granules with morphology intermediate between those of the two species. In our experiments, which involved the transfer of a single molecular species - the Osk^{imm} protein - rather than the more complex and less well defined polar plasm, we observed a more complete conversion of polar granules to the *D. immigrans* morphology. Specifically, when the only source of Osk was Osk^{imm}, all embryos at the end of the blastoderm stage had only a single large and irregular granule per pole cell, just as in *D. immigrans*. Our results reveal the central role of Osk in specifying the organization of the polar granules, demonstrating that Osk does have an ongoing role beyond the initial formation of polar granules. However, our results also raise the question of why Osk^{imm} alone was more effective in dictating a *D. immigrans* morphology than the combination of most or all required factors from *D. immigrans*. While we have no definitive answer, an obvious difference in the experiments is the absence of all endogenous Osk when we provided Osk^{imm} through a transgene. In the transplantation experiments there would initially be no appreciable level of *D. melanogaster* Osk in the anterior of the recipient embryos, as the programs of mRNA localization, translational regulation and protein anchoring very effectively limit Osk to the posterior pole of the embryo. However, less than a quarter of *osk* mRNA in early embryos is localized to the posterior pole (Bergsten and Gavis, 1999). Because Osk contributes to the localization and thus expression of its own mRNA, the ectopic anterior Osk^{imm} could promote accumulation of *D. melanogaster* Osk from unlocalized mRNA in the recipient embryos, which would then influence the morphology of the resulting

polar granules. We do observe polar granules with intermediate morphology when both Osk and Osk^{imm} are present at the posterior pole, consistent with this possibility.

Progression of polar granules through their development is accompanied by the appearance of nuclear bodies, whose striking similarity in structure has led to suggestions that they are related and that the nuclear bodies may in fact derive from polar granules (Mahowald et al., 1976). The evidence that nuclear bodies and polar granules have at least two shared components - Osk and Vas - demonstrates a close relationship. We have shown that Osk^{imm} is able to alter the morphology of *D. melanogaster* nuclear bodies, indicating that Osk plays a similar role in both structures.

Evidence of the central role of Osk in organizing polar granules and nuclear bodies focuses attention on how it performs these functions, and the question of what features of Osk determine which type of granule will form. The answer to the first question must involve the interactions of Osk with other granule components, but how molecular interactions of proteins whose volume is measured in Angstroms can direct formation of seemingly perfect spheres that are hundreds of nanometers in diameter is uncertain. The extreme regularity of the structures invites comparisons to other highly regular spherical structures, such as viral capsids. The principles underlying the assembly of icosahedral virions could also apply to spherical polar granules, with one important difference. The virions are assembled from a well defined number of subunits, usually organized as a series of hexamers (on each of the 20 faces) and pentamers (at the vertices of connecting each face) (Morgan, 2003). A similar assembly on the scale of polar granules would require subunits several orders of magnitude larger than the viral capsid proteins. Thus the subunits would have to consist of a very large number of individual polypeptides; these subunits could possibly correspond to the large number of small, sand-like polar granules seen very early in embryogenesis.

Lacking an understanding of how polar granules assemble, and of the precise role of Osk in this process, it is not surprising that a comparison of the amino acid sequences of Osk and Osk^{imm} does not suggest an explanation of why they specify different polar granule morphologies. Because one or more differences in molecular interactions is likely to be involved, we compared, by yeast two hybrid assays, the interactions of the two Osk proteins with the known *D. melanogaster* polar granule components, but found no substantial differences (data not shown). It may be that we did not evaluate the critical interaction, with a polar granule component not yet identified, or that significant differences can be subtle and will require a more quantitative assay to detect. Slight differences in the spacing or interaction between Osk and Osk^{imm} and other granule components could have a large effect on the overall structure, as hundreds or thousands of units come together. We also note the possibility that Osk and Osk^{imm} may have qualitative differences in their interactions. Extending the analogy to viral capsid assembly, the spherical polar granules could be comparable to wild type viral capsids, with the *D. immigrans*-like granules comparable to polymorphic aggregates of the coat proteins. While only the spherical assemblies could function to encase a viral genome, either type could contain integral germ cell determinants. Our results show that either type of polar granule can mediate germ cell formation in *D. melanogaster*.

MATERIALS AND METHODS

Production of *Drosophila immigrans* genomic library, cloning and sequencing of *osk^{imm}*

Genomic *Drosophila immigrans* DNA was partially digested with Sau3AI and ligated into the Lambda Fix II vector system (Stratagene). The resulting library was screened by

low stringency hybridization (McGinnis et al., 1984) with a 1.9 Kb EcoRI-EcoRI *osk* cDNA probe. The *osk^{imm}* gene sequence has been submitted to Genbank, accession number: DQ823083.

Oskar protein sequences from *D. virilis, D. pseudoobscura, D. ananassae, D. mojavensis, D. yakuba* and *A. gambiae* were all obtained from Flybase (http://species.flybase.net/) and compared using ClustalW (http://www.ebi.ac.uk/clustalw/). To form the graph, each amino acid was given a score from 0-8 depending on how many sequences were identical. The average of every 5 amino acids was plotted in Microsoft Excel.

Fly stocks and transgenes

D. immigrans flies were obtained from the Tucson Drosophila stock center. *P[uas-gfp-aub]* and *P[osk]* have been described previously (Harris and Macdonald, 2001, Kim-Ha et al., 1995). *P[vas-gfp]* flies were obtained from Anne Ephrussi.

To introduce *osk^{imm}* into *melanogaster* flies, a 8.7 Kb NotI-Asp718 genomic fragment containing the *osk^{imm}* transcription unit as well as 5 Kb upstream of the start codon, and 1Kb downstream of the polyadenylation site was inserted into a derivative of the pCaSpeR vector for P element transformation (Pirotta, 1988), modified by insertion of NotI and Asp718 linkers at blunt PstI and EcoRI sites, respectively. This transgene is referred to as *P[osk^{imm}]*. In addition, a transgene with the *osk^{imm}* 3'UTR and 3' flanking sequences replaced with a 1.6 kb HindIII to Asp718 fragment containing the *oskar* 3'UTR was inserted into the pCaSpeR vector. This transgene is referred to as *P[osk$^{imm3'mel}$]*.

The *P[M1L-Osk$^{imm3'mel}$]*, *P[M103L-Osk$^{imm3'mel}$]*, and *P[M103,106L Osk$^{imm3'mel}$]* transgenes were all modified from *P[osk$^{imm3'mel}$]* using the

QuikChange II XL site-directed mutagenesis kit (Stratagene). Microinjections were performed according to (Spradling and Rubin, 1982) into w^{1118} flies.

Antibody staining and confocal microscopy

Embryos were collected and stained as described previously (Macdonald and Struhl, 1986, Macdonald et al., 1991) using secondary antibodies coupled to Cy5 (Jackson Immunoresearch Laboratories). Osk antibodies were used at 1:3000, incubating at room temperature overnight. Stained embryos were mounted in Vectashield medium (Vector Labs) and imaged using a Leica TCS-SP confocal microscope.

In situ hybridization and Rnase protection assay

In situ hybridization was performed as described (Tautz and Pfeifle, 1989). The antisense osk^{imm} probe was synthesized from a 1.8 Kb XhoI to EcoRI fragment corresponding to the 5' portion of the osk^{imm} mRNA. This probe is specific for osk^{imm}. The probes were labeled with Digoxigenin conjugated nucleotides (Roche Diagnostic GmbH). RNase protection assays were performed as recommended by the manufacturer (RPA III kit, Ambion). The same probes as above were used, except that they were labeled with ^{32}P-UTP (NEN).

ACKNOWLEDGEMENTS

We thank Anne Ephrussi for providing the Osk antibody and the Tucson Drosophila Stock Center for providing *Drosophila immigrans* flies. We also thank Nan Yan for injecting *P[osk+]* and Mark Snee, Cuiyun Geng, and Daniel Jones for comments on the manuscript. This work was supported by a grant (GM54409) from the National Institutes of Health.

FIGURES

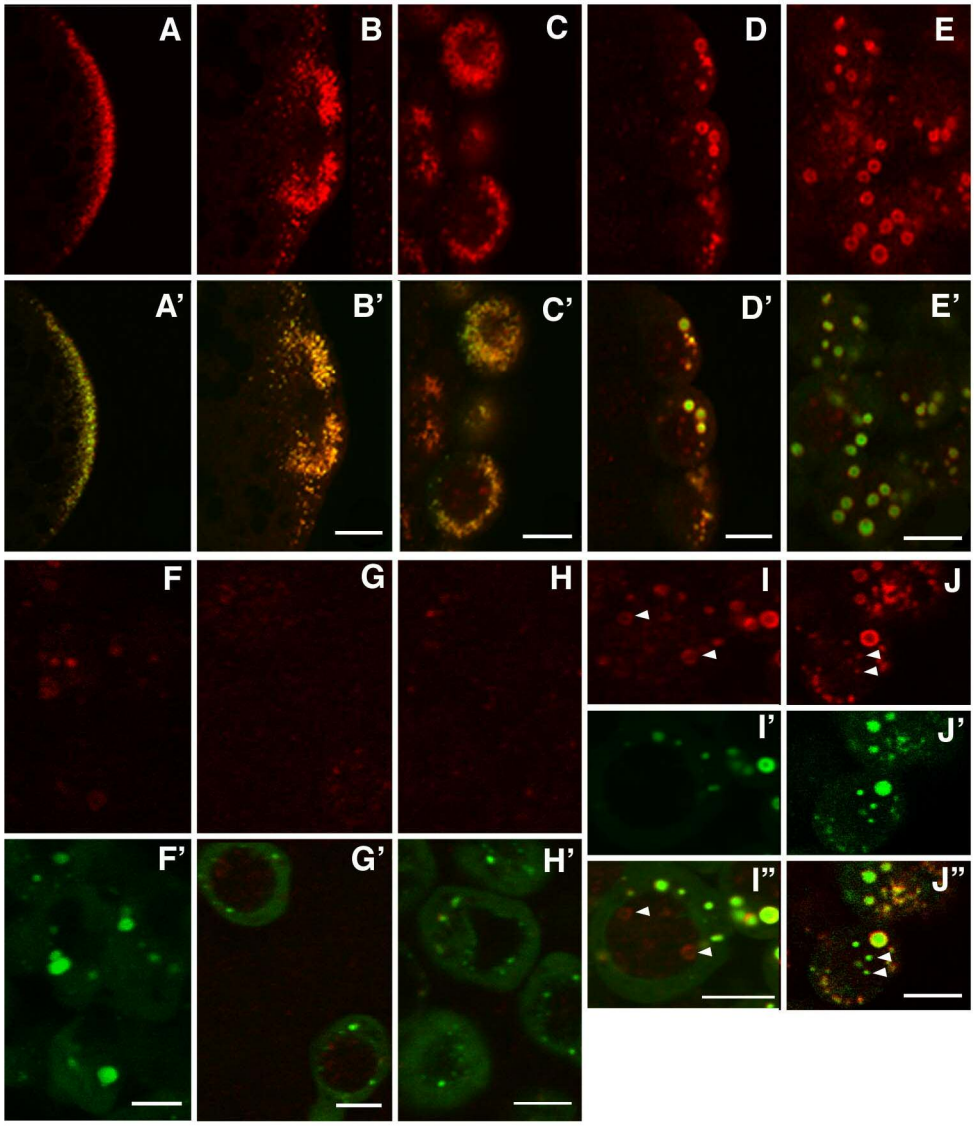


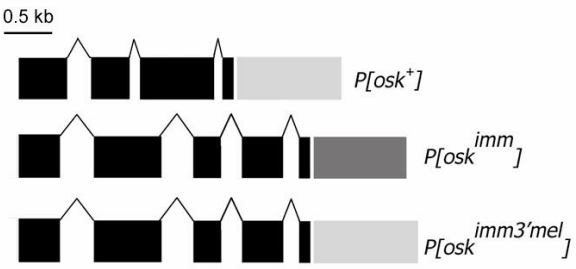
Figure 2.1: Oskar distribution during pole cell development and migration.

A-H. Detection of Osk in embryos from egg lay (A) to arrival of the pole cells at the gonad (H). Paired panels (e.g. A and A') show Osk in A-H and double labeling of Osk (red) and GFP-Aub (green) in A'-H'. GFP-Aub is strictly cytoplasmic, and its absence demarcates nuclei after pole cells have formed. In early embryos (egg lay, A; pole bud

Figure 2.1: Oskar distribution during pole cell development and migration (contd.).

formation, B; pole cell formation, C), Osk is cytoplasmic in small particles, and also appears in nuclei starting at syncytial blastoderm stage (C). Panels A and A' show the entire posterior end of the embryo, while all other panels focus on a portion of the extreme posterior covering several pole cells. During cellular blastoderm (D) and gastrulation (E) stages, Osk appears in the periphery of spheres in the cytoplasm. By early migration of the pole cells (F), Osk begins to be harder to detect. In pole cells during later migration (G) and arrival at the gonad (H), Osk is difficult to detect and only appears in small foci. Note that in F'-H', cytoplasmic polar granules are still detectable by GFP-Aub and have fragmented into smaller particles. I-I''. Nuclear bodies of pole cells at gastrulation, stained for Osk (I), GFP-Aub (I') and merge (I''). Arrows point to the nuclear bodies, which do not contain GFP-Aub, as previously noted (Harris and Macdonald, 2001). J-J''. Polar granules and nuclear bodies are also detectable using Vasa-GFP (J'), which colocalizes with Osk (J), merge (J''). All scale bars are 5 μm.

A



B

Genotype*	Cuticle phenotype (%)				
	Wildtype	Anterior defects	Bicaudal	Posterior defects	n
w^{1118}	100.0	0.0	0.0	0.0	217
osk^{54}/Df(3R)pXT-103	0.0	0.0	0.0	100.0	44
P[osk]-1/+; osk^{54}/Df(3R)pXT-103	93.8	6.2	0.0	0.0	173
P[osk]-2/+; osk^{54}/Df(3R)pXT-103	98.4	1.6	0.0	0.0	126
P[$osk^{imm}3^{mel}$]-1/+; osk^{54}/Df(3R)pXT-103	52.9	23.3	23.8	0.0	198
P[$osk^{imm}3^{mel}$]-2/+; osk^{54}/Df(3R)pXT-103	75.6	24.4	0.0	0.0	164
P[osk^{imm}]-1/+; osk^{54}/Df(3R)pXT-103	87.5	12.5	0.0	0.0	168
P[osk^{imm}]-2/+; osk^{54}/Df(3R)pXT-103	60.3	39.7	0.0	0.0	209

C

Genotype*	Pole cell No. Nuclear cycle 14	n
w^{1118}	36.2	25
osk^{54}/Df(3R)pXT-103	0.0	21
P[osk]-1/+; osk^{54}/Df(3R)pXT-103	23.6	36
P[osk]-2/+; osk^{54}/Df(3R)pXT-103	22.5	24
P[$osk^{imm}3^{mel}$]-1/+; osk^{54}/Df(3R)pXT-103	10.7	32
P[$osk^{imm}3^{mel}$]-2/+; osk^{54}/Df(3R)pXT-103	5.7	21
P[osk^{imm}]-1/+; osk^{54}/Df(3R)pXT-103	7.6	28
P[osk^{imm}]-2/+; osk^{54}/Df(3R)pXT-103	5.9	33

*All genotypes are that of the mother.

Figure 2.2: Body patterning and pole cell forming activities of *osk* and osk^{imm} transgenes in *D. melanogaster*. A. Exon/intron organization of *osk* and osk^{imm} genes. The coding regions are darkly shaded, and the 3'UTR's are in a lighter shade. Both *P[osk+]* and *P[*osk^{imm}*]* contain their own coding region and 3' UTR, while the *P[*$osk^{imm3'mel}$*]* transgene contains the osk^{imm} coding region and *osk* 3'UTR. The exon junctions of osk^{imm} were confirmed by sequencing osk^{imm} cDNA from *D. immigrans* embryos. B and C. Quantitation of body patterning (B) and pole cell forming (C) activities of *osk* and osk^{imm} transgenes.

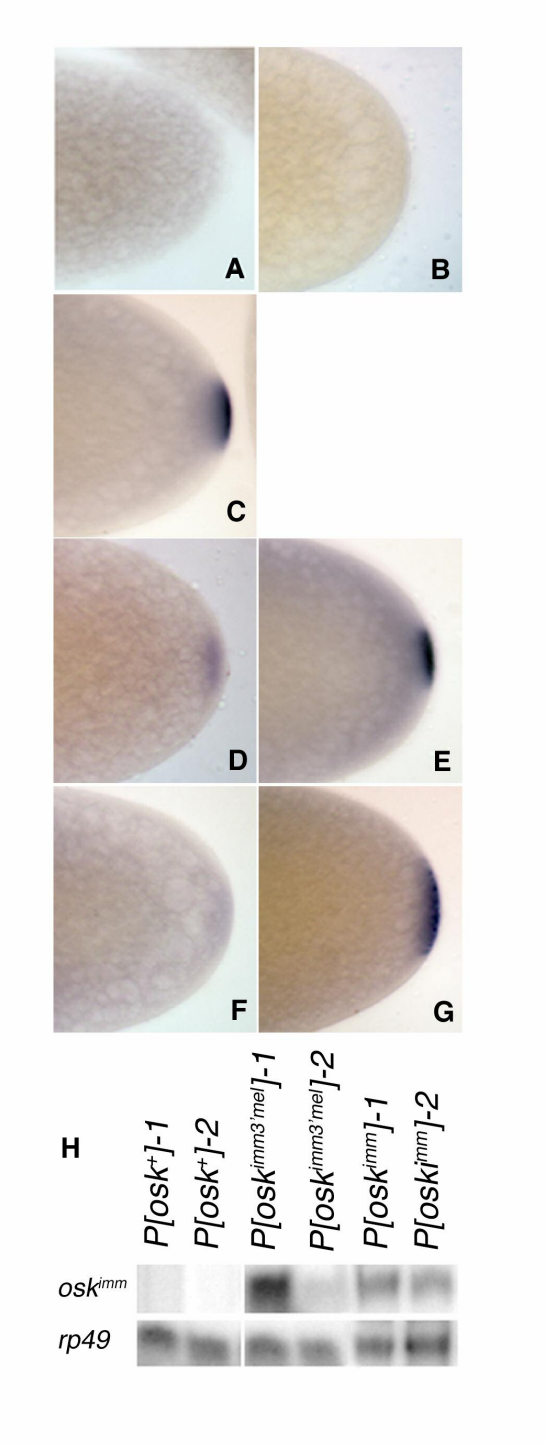


Figure 2.3: Embryonic localization of *osk* and *osk^{imm}* mRNAs.

A-G. Embryos probed with an *osk* RNA probe (left panels) or an *osk^{imm}* RNA probe (right panels). Panel A, *osk^{54}/Df* embryo, showing absence of *osk* mRNA localization (Ephrussi et al., 1991, Kim-Ha et al., 1991). Panel B, *w^{1118}* embryo; the absence of signal from the localized *osk* mRNA demonstrates the absence of cross hybridization between *osk* and *osk^{imm}* mRNAs. Panels C, D and F, detection of endogenous *osk* mRNA in *P[osk^{+}]* (C), *P[osk$^{imm3'mel}$]* (D), or *P[osk^{imm}]* (F) embryos. Panels E and G, detection of transgenic *osk^{imm}* mRNA in *P[osk$^{imm3'mel}$]* (E) or *P[osk^{imm}]* (G) embryos. H. RNase protection assay used to detect the level of *osk^{imm}*expression in each representative transgenic line. The *osk^{imm}*probe is specific for *osk^{imm}*, as there is no band detected in lanes 1 and 2. The *rp49* mRNA was detected as a loading control.

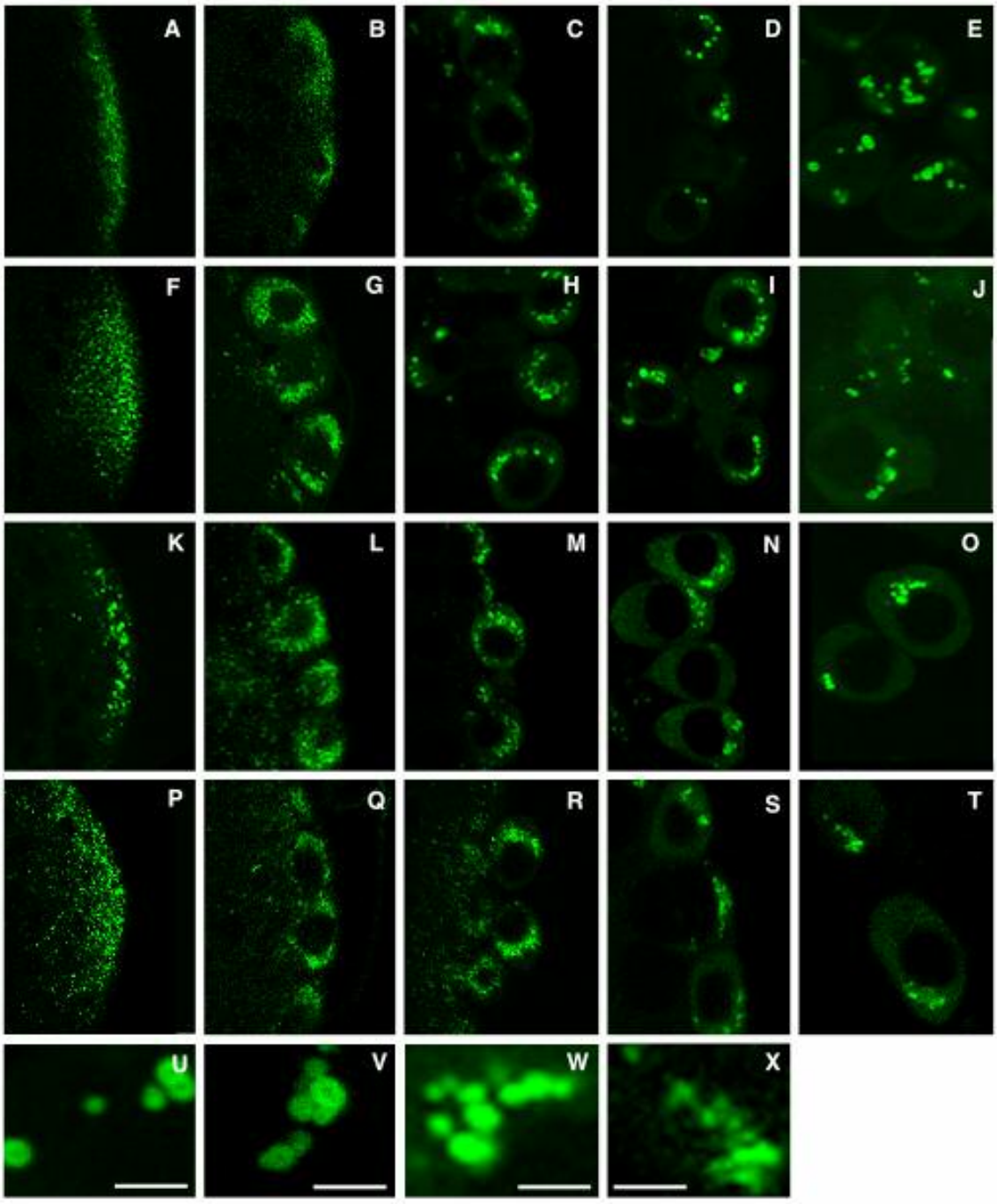


Figure 2.4: Osk^{imm} dictates polar granule morphology.

All panels show signal from GFP-Aub. Panels are arranged so that from left to right, each group of five shows granule morphology at egg lay, pole bud formation, syncytial blastoderm, cellular blastoderm and gastrulation. Panels A, F, K and P are at lower

Figure 2.4: Osk^{imm} dictates polar granule morphology (contd.).

magnification to show the majority of the polar plasm. Panels U-X are enlargements of the granules in panels E, J, O and T, respectively. Maternal genotypes: A-E, U, *osk^{54}/+*; F-J, V, *P[osk^{+}]/+; osk54/Df*; K-O, W, *P[osk^{imm}]/+; osk^{54}/Df*; P-T, X, *P[osk$^{imm3'mel}$]/+; osk^{54}/Df* .

In early embryos, the granules of all genotypes appear essentially the same: small, sand-like and spread throughout the cytoplasm. At cellular blastoderm and gastrulation, polar granules in control embryos with either a single endogenous copy of *osk^{+}* (D, E) or no endogenous *osk* and a single copy of the *P[osk^{+}]* transgene (I,J) have the characteristic wild type 'donut' appearance. In *P[osk^{imm}]/+; osk^{54}/Df* (N, O) or *P[osk$^{imm3'mel}$]/+; osk^{54}/Df* (S, T) embryos, the granules fail to form 'donuts' and fuse into one area of granule material per cell. When seen as serial projections, these areas of granule material appear as a single continuous aggregate. Scale bar is 2 μm for U-X.

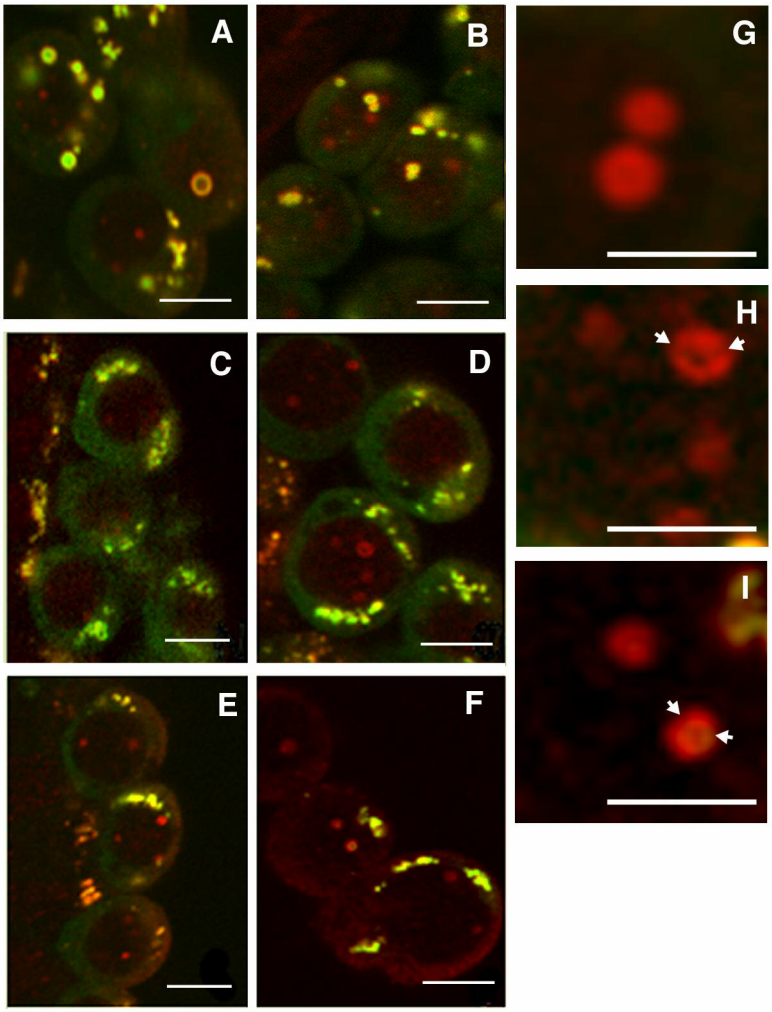
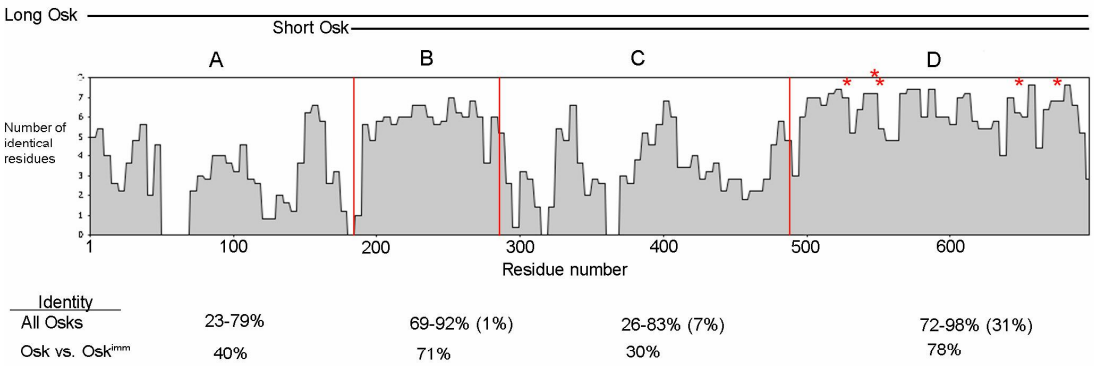


Figure 2.5: Osk^{imm} dominantly influences polar granule and nuclear body morphology.

For all panels green signal is GFP-Aub and red is Osk. All embryos are from otherwise wild type mothers bearing a single copy of the following transgenes: A, B and G, *P[osk^{+}]* ; C, D and H *P[osk^{imm}]*; E, F and I, *P[osk$^{imm3'mel}$]*. Panels A-F show pole cells from cellular blastoderm (A, C, E) or gastrulation (B, D, F) stage embryos. Panels G-I show portions of pole cell nuclei from gastrulating embryos. *P[osk^{+}]* polar granules are spherical (A and B). In the presence of Osk^{imm} expressed with its own 3' UTR (C and D) or with the *D. melanogaster osk* 3' UTR (E and F), the polar granules fuse into one to three large aggregates. Nuclear bodies are normally spherical (G), but in *P[osk^{imm}]* (H) or*[osk$^{imm3'mel}$]* (I) embryos the nuclear bodies show deformations and discontinuities (arrows). Scale bars: A-F, 5 µm; G-I, 2 µm.

A.



B.

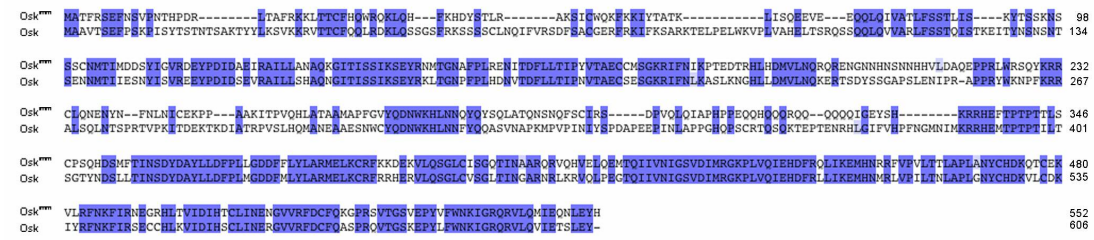


Figure 2.6: Conservation of Osk amino acid sequences.

A. Graphic representation of the identity between Osk^{imm} and 6 other *Drosophila* Osk proteins, as well as Osk from *A. gambiae*. The graph is divided into four sections, according to regions of high or low homology. Stars indicate the position of missense mutations [*osk*^{301}, *osk*^{255}, osk^{6B10}, *osk*^{88} and *osk*^{166}, respectively, (Breitwieser et al., 1996, Kim-Ha et al., 1991)]**.** Above the graph, long and short Osk are depicted as they relate to the regions in the graph. Below the graph, specific ranges of amino acid sequence identity are shown. The percentages in parentheses refer to the identity of *A. gambiae* as compared to the other Osk sequences. B. Direct comparison of the sequences of Osk^{imm} and Osk. Identical residues are shaded.

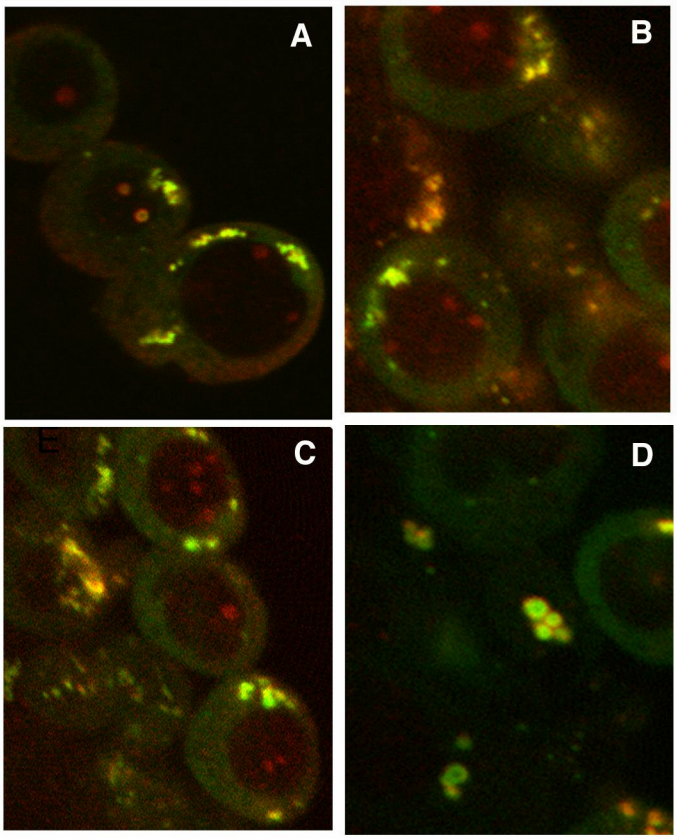


Figure 2.7: Short, but not Long, Osk^{imm} controls polar granule morphology.

Polar granule and nuclear body morphology in gastrulating embryos. Green signal is GFP-Aub, and red is Osk. All embryos are from otherwise wild type mothers bearing a single copy of the following transgenes: A, *P[osk$^{imm3'mel}$]*; B, *P[M1L-osk$^{imm3'mel}$]*, C, *P[M103L-osk$^{imm3'mel}$]*; D, *P[M103,106L osk$^{imm3'mel}$]*. Polar granule morphology is shifted towards the *D. immigrans* type in A-C, but not D. While there are spherical bodies in A-C, they are all in nuclei (i.e. nuclear bodies), and the cytoplasmic polar granules are not spherical. Scale bar is 5 µm.

REFERENCES

Arkov, A. L., J. Y. Wang, A. Ramos, and R. Lehmann. 2006. The role of Tudor domains in germline development and polar granule architecture. *Development.*

Bardsley, A., K. McDonald, and R. E. Boswell. 1993. Distribution of tudor protein in the *Drosophila* embryo suggests separation of functions based on site of localization. *Development.* 119:207-219.

Bergsten, S. E., and E. R. Gavis. 1999. Role for mRNA localization in translational activation but not spatial restriction of *nanos* RNA. *Development.* 126:659-669.

Boswell, R. E., and A. P. Mahowald. 1985. *tudor*, a gene required for assembly of the germ plasm in Drosophila melanogaster. *Cell.* 43:97-104.

Breitwieser, W., F.-H. Markussen, H. Horstmann, and A. Ephrussi. 1996. Oskar protein interaction with vasa represents an essential step in polar granule assembly. *Genes Dev.* 10:2179-2188.

Cerutti, L., N. Mian, and A. Bateman. 2000. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the piwi domain. *Trends Biochem Sci.* 25:481-482.

Counce, S. J. 1963. Developmental morphology of polar granules in Drosophila including observations on pole cell behavior and distribution during embryogenesis. *J. Morph.* 112:129-145.

Ephrussi, A., L. K. Dickinson, and R. Lehmann. 1991. *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell.* 66:37-50.

Ephrussi, A., and R. Lehmann. 1992. Induction of germ cell formation by *oskar*. *Nature.* 358:387-392.

Geigy, R. 1931. Action de l'ultra-violet sur le pole germinale dans l'oeuf de Drosophila melanogaster. *Rev. Suisse Zool.* 38:187-288.

Gunkel, N., T. Yano, F. H. Markussen, L. C. Olsen, and A. Ephrussi. 1998. Localization-dependent translation requires a functional interaction between the 5' and 3' ends of oskar mRNA. *Genes Dev.* 12:1652-1664.

Harris, A. N., and P. M. Macdonald. 2001. Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development.* 128:2823-2832.

Hay, B., L. Y. Jan, and Y. N. Jan. 1988. A protein component of *Drosophila* polar granules is encoded by *vasa* and has extensive sequence similarity to ATP-dependent helicases. *Cell.* 55:577-587.

Illmensee, K., and A. P. Mahowald. 1974. Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. *Proc. Natl. Acad. Sci. USA.* 71:1016-1020.

Kennerdell, J. R., S. Yamaguchi, and R. W. Carthew. 2002. RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. *Genes Dev.* 16:1884-1889.

Kim-Ha, J., J. L. Smith, and P. M. Macdonald. 1991. *oskar* mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell.* 66:23-35.

Kim-Ha, J., K. Kerr, and P. M. Macdonald. 1995. Translational regulation of *oskar* mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell.* 81:403-412.

Kim-Ha, J., P. J. Webster, J. L. Smith, and P. M. Macdonald. 1993. Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development.* 119:169-178.

Lasko, P. F., and M. Ashburner. 1988. The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. *Nature.* 335:611-617.

Lasko, P. F., and M. Ashburner. 1990. Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. *Genes Dev.* 4:905-921.

Lehmann, R., and C. Nüsslein-Volhard. 1986. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in Drosophila. *Cell.* 47:141-152.

Liang, L., W. Diehl-Jones, and P. Lasko. 1994. Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. *Development.* 120:1201-1211.

Macdonald, P. M., S. K.-S. Luk, and M. Kilpatrick. 1991. Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes Dev.* 5:2455-2466.

Macdonald, P. M., and G. Struhl. 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature.* 324:537-545.

Mahowald, A. P., K. Illmensee, and F. R. Turner. 1976. Interspecific transplantation of polar plasm between Drosophila embryos. *J Cell Biol.* 70:358-373.

Mahowald, A. P. 1962. Fine structure of pole cells and polar granules in *Drosophila melanogaster*. *J. exp. Zool.* 151:201-216.

Mahowald, A. P. 1968. Polar granules of *Drosophila*. II. Ultrastructural changes during early embryogenesis. *J. exp. Zool.* 167:237-262.

Mahowald, A. P. 1971. Polar granules of *Drosophila*. III. The continuity of polar granules during the life cycle of *Drosophila*. *J. exp. Zool.* 176:329-344.

Markussen, F.-H., A.-M. Michon, W. Breitwieser, and A. Ephrussi. 1995. Translational control of *oskar* generates Short OSK, the isoform that induces pole plasm assembly. *Development.* 121:3723-3732.

McGinnis, W., M. S. Levine, E. Hafen, A. Kuroiwa, and W. J. Gehring. 1984. A conserved DNA sequence in homoeotic genes of the Drosophila Antennapedia and bithorax complexes. *Nature.* 308:428-433.

Megosh, H. B., D. N. Cox, C. Campbell, and H. Lin. 2006. The Role of PIWI and the miRNA Machinery in Drosophila Germline Determination. *Curr. Biol.* In press

Morgan, G. J. 2003. Historical review: Viruses, crystals and geodesic domes. *Trends Biochem Sci.* 28:86-90.

Munro, T. P., S. Kwon, B. J. Schnapp, and D. St Johnston. 2006. A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. *J Cell Biol.* 172:577-588.

Nakamura, A., R. Amikura, M. Mukai, S. Kobayashi, and P. F. Lasko. 1996. Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. *Science.* 274:2075-2079.

Pirotta, V. 1988. Vectors for P-mediated transformation in Drosophila. *In* Vectors: A survey of molecular cloning vectors and their uses, Rodriguez, R. L., and D. T. Denhardt, editors. Butterworths, Boston. 437-456.

Rongo, C., E. R. Gavis, and R. Lehmann. 1995. Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development.* 121:2737-2746.

Saffman, E. E., and P. Lasko. 1999. Germline development in vertebrates and invertebrates. *Cell Mol Life Sci.* 55:1141-1163.

Schüpbach, T., and E. Wieschaus. 1986. Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Dev. Biol.* 195:302-317.

Smith, J. L., J. E. Wilson, and P. M. Macdonald. 1992. Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. *Cell.* 70:849-859.

Spradling, A. C., and G. M. Rubin. 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. *Science.* 218:341-347.

Tautz, D., and C. Pfeifle. 1989. A non radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals a translational control of the segmentation gene hunchback. *Chromosoma.* 98:81-85.

Thomson, T., and P. Lasko. 2004. Drosophila tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. *Genesis.* 40:164-170.

Vanzo, N. F., and A. Ephrussi. 2002. Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. *Development.* 129:3705-3714.

Webster, P. J., J. Suen, and P. M. Macdonald. 1994. *Drosophila virilis oskar* transgenes direct body patterning but not pole cell formation or maintenance of mRNA localization in *D. melanogaster*. *Development.* 120:2027-2037.

Wilson, J. E., J. E. Connell, and P. M. Macdonald. 1996. *aubergine* enhances *oskar* translation in the *Drosophila* ovary. *Development.* 122:1631-1639.

Chapter Three: Bluestreak, a novel protein involved in pole cell behavior during migration, associates with centrosomes and the nuclear membrane in pole cells

ABSTRACT

During embryogenesis, the germ cells (pole cells) of the *Drosophila* embryo migrate from the posterior pole through a complex route to reach their final destination, the gonad. The pole cells are the first cells to form in the embryo, and they retain a round and regular shape until gastrulation. As they migrate, the pole cells extend pseudopodia in response to guidance cues, enabling them to move between and around the somatic cells of the embryo. Here we report the identification of Bluestreak (Blue), a novel protein involved in pole cell migration. The pole cells of embryos from mothers mutant in *Bluestreak* display defects in morphology, beginning in the syncytial blastoderm stage. These defects in shape resemble pseudopodia, and we show that mutations in genes required for guiding pole cells to the somatic cells of the gonad – *fpps*$^{-}$, *quemao*$^{-}$ and *columbus*$^{-}$, dominantly suppress the *Blue*$^{-}$ pole cell phenotype of early embryos. We further show that Blue colocalizes with γ-tubulin to centrosomes in cells throughout the early embryo, and localizes to the nuclear membrane in pole cells. Our evidence suggests that Blue may act to regulate the behavior of pole cells during migration in *Drosophila*.

INTRODUCTION

The formation of germ cells distant from their final location in the mature organism is common in development. In *Drosophila melanogaster*, germ cells form at the posterior pole of the embryo very early in embryogenesis, and must migrate through the embryo in order to associate with specific somatic cells to form the gonad. Investigation of this process has identified a variety of required proteins, several of which have also

been shown to play a role in other migration processes, such as neurogenesis and the metastatic behavior of cancer cells (Brown, 2006, Deshpande, 2001, Li, 2003).

The process of germ cell formation begins in the oocyte, when a specialized cytoplasm, termed germ plasm, is formed at the posterior pole. Germ plasm formation is dependent on the concentrated localization of Oskar, which recruits further proteins and RNAs to the germ plasm to produce germ cell specific structures known as polar granules (Breitwieser et al., 1996, Ephrussi and Lehmann, 1992, Ephrussi et al., 1991, Vanzo and Ephrussi, 2002). The germ plasm remains at the posterior pole in the early embryo, which develops as a syncytium. Several rounds of nuclear division occur in the middle of the embryo without cytokinesis. As the nuclei approach the periphery of the egg, a few nuclei will enter the germ plasm and bud off from the rest of the embryo, creating the germ cells (pole cells). The pole cells and somatic nuclei will continue to divide three more times, after which the somatic cells of the embryo cellularize via membrane invagination. Soon after cellularization, the embryo will begin gastrulation and the pole cells will begin their journey through the embryo to eventually associate with the somatic cells of the gonad (Campos-Ortega, 1985).

Germ cell migration in *Drosophila* occurs in several stages, and mutants with defects at many of these stages have been identified (for a review, see Santos (2004a). Migration begins at gastrulation, as the pole cells are carried into the embryo along the midgut primordium epithelium (PMG) (Rabinowitz, 1941, Sonnenblick, 1941). Even though they are pulled into the embryo passively, the pole cells already show signs of migratory behavior, since evidence of pseudopodia has been detected at this stage (Jaglarz and Howard, 1995). Next, the pole cells will traverse the PMG and begin to migrate towards the mesoderm. Mutations in *trapped-in-endoderm1* (*tre-1*) affect the migration of the pole cells at this stage (Kunwar, 2003). *tre1* is a G-protein coupled

receptor expressed in the pole cells, and the pole cells of *tre1*$^{\Delta EP5}$ mutant embryos are unable to exit the PMG. Migration through the PMG is also dependent on changes within the epithelium (Callaini, 1995, Jaglarz, 1994), as mutants in *serpent* and *huckebein*, which affect the dissolution of apical junctions between the cells in the PMG, also prevent exit of the pole cells (Jaglarz and Howard, 1995, Moore et al., 1998).

Once the pole cells exit the PMG, they will migrate dorsally and separate into two groups that associate with the lateral mesoderm. The lipid phosphate phosphatases, Wunen and Wunen2, are required in both the pole cells and the soma, and affect the migration and survival of the pole cells as they migrate to the mesoderm (Hanyu-Nakamura, 2004, Renault et al., 2004, Sano, 2005, Starz-Gaiano et al., 2001, Zhang et al., 1997, Zhang, 1996). In the soma, the Wunens are expressed in the ventral portion of the PMG and in the central nervous system, acting to repel the pole cells dorsally and aiding in their bifurcation. The expression of Wunen and Wunen-2 in the pole cells affect their survival, so that any left in the middle of the embryo after exit from the midgut are eliminated.

The final step in migration is the arrival of the pole cells at the somatic gonadal precursors (SPGs). These cells make up the somatic portion of the adult gonad. The attraction of the pole cells to the SGPs has been shown to be controlled by the enzyme 3-Hydroxy 3-Methylglutaryl Coenzyme A Reductase (HMGCoAR or *Hmgcr* (*columbus, clb*). *clb* is expressed throughout the mesoderm as the pole cells exit the midgut, and is restricted to the SGPs as embryogenesis progresses (Van Doren et al., 1998). In embryos mutant for *clb*, pole cells are scattered throughout the embryo and few if any associate with the SGPs. In addition, ectopic expression of *clb* is sufficient to attract the pole cells to the source of expression, suggesting that *clb* is required for the production of a germ cell attractant. There are two main biosynthetic pathways downstream of HMGCoAR, the

production of cholesterol and of isoprenoids. Mutations in *fpps, quemao,* and *βGGT1*, which encode enzymes of the isoprenoid biosynthesis pathway, also display defects in germ cell migration. This indicates that it is the isoprenoid pathway downstream of HMGCoAR which produces the germ cell attractant, although the nature of the attractant is still unknown (Santos, 2004b).

The Jak/Stat signaling pathway also affects the migration of primordial germ cells in response to Torso signaling (Brown, 2006, Li, 2003). Torso is a receptor tyrosine kinase, and a member of the terminal group of maternal effect genes, which are required for the proper patterning of the anterior and posterior termini of the embryo (Duffy, 1994). Li et al. (2003) went on to show that Torso signaling activates Stat in the pole cells of early embryos, and that this signaling cascade was important for pole cell proliferation and migration. Constitutive activation of Tor, or of the downstream JAK, Hop, leads to an overproliferation of pole cells which exit the midgut early and migrate errantly through the mesoderm. Loss-of-function mutations in *tor* render the pole cells immotile, and the pole cells are found outside of the mature embryo. These embryos also lack a midgut primordium, raising the possibility that the lack of this epithelium to initially carry the pole cells into the embryo compounds the phenotype (Hou, 1995, Klinger et al., 1988).

Here we report our analysis of *Bluestreak* (Blue), a novel protein involved in germ cell migration. The pole cells of embryos from either heterozygous or homozygous *Blue*$^{-}$ mothers are misshapen in early embryos, and continue to display an extraordinary morphology as they migrate. By the time normal germ cells associate with the SGPs, embryos from homozygous or hemizygous *Blue*$^{-}$ mothers have few to no pole cells in the gonads. We have found that mutations in enzymes of the isoprenoid pathway - *fpps, clb* and *qm* - dominantly suppress the *Blue*$^{-}$ pole cell phenotype, suggesting that Blue may

act to regulate the response of the pole cells to this pathway. We also show that Bluestreak colocalizes with γ-tubulin to centrosomes throughout embryo, and localizes to the nuclear membrane in pole cells. Our analysis lends new insights into the mechanisms controlling pole cell migration, and we speculate about the possible mechanism by which Blue acts in this process.

RESULTS

Embryos from heterozygous *Blue*$^{-}$ mothers display a dominant pole cell phenotype

The *Drosophila* embryo initially develops as a syncytium, in which the zygotic nucleus undergoes 13 rounds of nuclear division without cytokinesis. Beginning at the seventh round of division, the majority of the nuclei move toward the surface of the embryo to form the syncytial blastoderm. At the tenth round, between 12-14 cells pinch off at the posterior pole, these pole cells are the germ cells of the embryo. Both the pole cells and the rest of the nuclei in the embryo will continue to divide, although not in synchrony with each other, until the 14th nuclear cycle, when the somatic cells of the embryo cellularize. Soon after cellularization, gastrulation begins as the ventral furrow ingresses and the midgut primordium pulls the pole cells anterodorsally (Campos-Ortega, 1985). Up until gastrulation, the pole cells are regular and spherical, however as gastrulation ensues, they begin to extend projections, presumably in preparation for migration (Jaglarz and Howard, 1995).

While the pole cells of stage 4 and 5 (syncytial blastoderm and cellular blastoderm, respectively) embryos from wildtype mothers are spherical (Fig 3.1A), embryos of these stages from *Df(3L)fz-GF3b*/+ mothers show defects in pole cell shape, with 68.8% of embryos having pole cells of odd or extended shape (Fig 3.1B). Since

Df(3L)fz-GF3b is a large deficiency (Fig 3.2A), we tested smaller deficiencies in the region to narrow down the gene causing the defect. Of all the deficiencies tested, only embryos from *Df(3L)fz-GF3b/TM6b, Df(3L)ED4543/TM3* and *Df(3L)Blue^{19b}/TM6b* (Fig 3.1B, C, D) mothers exhibited the phenotype. The smallest deficiency, *Df(3L)Blue^{19b}*, deletes only five genes: *Hsc70Cb, Bluestreak (Blue), CG6833, CG13484,* and *CG32138.* Since we also found that embryos from mothers carrying the P-element, *P[EY12221]* (hereafter referred to as *Blue^{1}*) show the phenotype (Fig 3.1G) and since this P-element resides between the promoter and transcription start site for *Blue* (Bellen et al., 2004), we focused our attention on investigation of *Blue.*

While embryos from *Blue^{1}/ Blue^{1}* mothers have misshapen pole cells, it was possible that this mutation was hypomorphic, since only 14.5% of embryos display the phenotype (Table 3.1). To obtain stronger mutants in *Blue,* we used imprecise excision and obtained two new mutants: *Blue^{2}* and *Blue^{3}*. *Blue^{2}* retains a 423 bp portion of the P-element, deleting no portion of the *Blue* locus. *Blue^{3}* deletes a 909 bp region in the locus, deleting the transcription start site as well as 278 bp of the coding region, including exons 1 and part of exon 2 (Fig 3.2B). 79.3% of embryos from *Blue^{2}*/+ mothers and 88.2% of embryos from *Blue^{3}*/+ mothers show the misshapen phenotype (Fig 3.1H, I). This percentage does not change significantly when *Blue^{2}*/+ mothers are mated to wildtype fathers (73.7%, Table 3.4), and is not present in embryos from wildtype mothers mated to *Blue^{2}/ Blue^{2}* fathers, indicating that the gene acts maternally and the mutant phenotype is dominant. We will therefore refer to embryos from heterozygous or homozygous *Blue^{-}* mothers according to the genotype of the mother. To further demonstrate that the pole cell phenotype is a consequence of mutations affecting *Blue*, we made a genomic construct (*P[Blue^{+}]*), which includes the entire *Blue* coding region as well as 1.6 Kb upstream of the start codon and 0.4 Kb downstream of the poly A site, but does not

contain any of the coding region of the genes flanking *Blue* (Fig 3.2B). This transgene is able to rescue the misshapen pole cell phenotype of $Blue^2$/+ embryos (Fig 3.1J, Table 3.1).

The $Blue^2$ allele is homozygous viable, and $Blue^2$/ $Blue^2$ flies do not show defects in morphology or behavior. The $Blue^3$ allele, although not homozygous viable, is viable over a deficiency, suggesting that the chromosome contains a second site mutation causing the lethality. $Blue^3$/+ and $Blue^3$/*Df* flies are also free of overt defects in adult morphology and behavior. The most consistent and severe defects in heterozygous, homozygous or hemizygous $Blue^-$ embryos are in pole cell formation and migration. The vast majority of $Blue^-$ embryos cellularize properly and progress normally through gastrulation, although a small percentage of cuticles show germ band elongation defects ($Blue^2$/$Blue^2$ (13.1%, n = 61) and $Blue^3$/+ (9.0%, n = 89)).

The odd extensions of the pole cells in stage 5 $Blue^-$ embryos are substantially abnormal compared to the pole cells of wildtype embryos at this stage. These extensions could be blebbing, a phenomenon associated with programmed cell death (Mills, 1998). To determine whether the pole cells were dying, we stained embryos for the active form of Caspase-3, which is detectable in cells undergoing apoptosis (Brennecke et al., 2003, Yu, 2002). However, we did not detect Caspase-3 staining in any of the misshapen pole cells (Fig 3.3B). To determine if our staining conditions were adequate to detect Caspase activity, we took advantage of the fact that apoptosis can be induced by overexpression of the *head involution defective* (*hid*) gene (Grether, 1995). Flies carrying a construct expressing *hid* under the control of a heat shock promoter, *P[hs-hid],* produce embryos which upon heat shock show high levels of programmed cell death throughout the embryo. These embryos were positive for Caspase-3 using our staining conditions (Fig 3.3C), indicating that the absence of staining in $Blue^-$ embryos is due to lack of active

Caspase in the pole cells. We also used Topro-3 to stain the DNA in the pole cells, since the nuclei of cells undergoing apoptosis can show signs of chromatin condensation (Saraste, 2000). Figure 3.3D shows wildtype pole cells, and the nucleus in each cell is uniformly round, even in dividing cells (arrows in Fig 3.3D'). In *Blue*^{2}/+ embryos, we detect pole cells that have extensions that are devoid of DNA, yet the nucleus in the main body of the cell is intact and similar in morphology and size to wildtype (Fig 3.3E). This is the case for all misshapen pole cells in this genotype. We were able to find pole cells in *Blue*^{2}*/Df(3L)ED4543* embryos which have two nuclei in one cell, however, even in this genotype, the nuclei are round and the extensions of misshapen pole cells do not contain DNA (Fig 3.3F). These results suggest that the misshapen phenotype of pole cells in stage 5 *Blue*$^{-}$ embryos is not due to programmed cell death.

Adult female progeny from *Blue*$^{-}$ mothers can be agametic

In addition to the pole cell phenotype at stage 5, we also noted that adult daughters of *Blue*$^{-}$ mothers can be agametic (Table 3.2). To explore when this reduction in germ cells occurs, we stained both wildtype and *Blue*$^{-}$ embryos at various stages of development with the pole cell specific antibody, α-Vasa, to count and track the pole cells (Hay et al., 1988). In wildtype embryos, an average of 36.2 spherical pole cells form by stage 5. These cells will migrate without further division (Campos-Ortega, 1985, Underwood et al., 1980) and approximately 15.5 pole cells will populate each gonad by stage 15. In *Blue*^{2}/+, *Blue*^{2}*/Blue*^{2} or *Blue*^{2}*/Df(3L)ED4543* embryos, though, the number of pole cells at stage 5 and at stage 15 is reduced (Table 3.3). Stage 15 *Blue*^{2}*/Blue*^{2} or *Blue*^{2}*/Df(3L)ED4543* embryos have few to no pole cells in either one or both gonads. Furthermore, no pole cells are found in ectopic positions in the embryo (Fig 3.4F). It is possible that the low number of pole cells found in stage 15 *Blue*$^{-}$ embryos is only due to

an initial reduction in the number of pole cells formed and normal pole cell losses during migration (Coffman, 2002). We did indeed find that fewer pole cells are formed initially in *Blue*$^{-}$ embryos, and that this reduction can be traced to defects during oogenesis (see below).

If the reduction in pole cells in stage 15 embryos is the result of the reduction in pole cells in stage 5 embryos, then increasing the amount of pole cells early should increase the amount of pole cells at later stages. Increasing the amount of *oskar* has been shown to increase the number of pole cells in the embryo (Ephrussi and Lehmann, 1992, Smith et al., 1992). We therefore counted pole cells in stage 5 and stage 15 embryos from *Blue*$^{-}$ mothers carrying a copy of an *oskar* transgene (*P[osk*$^{+}$*]/+; Blue*^{2}*/+)* (Table 3.3). In *P[osk*$^{+}$*]/+; Blue*^{2}*/+* embryos, the number of pole cells formed at stage 5 is increased from an average of 25.8 pole cells per *Blue*^{2}*/+* embryo to 49.6 pole cells per *P[osk+]/+; Blue*^{2}*/+* embryo (Table 3.3). Despite the dramatic increase in pole cell number at stage 5, the average number of pole cells per gonad in stage 15 embryos is still below wildtype (15.5 ± 1.7 in wildtype and 12.5 ± 2.8 in *P[osk*$^{+}$*]/+; Blue*^{2}*/+*). This difference is statistically significant ($p < .001$), and indicates that an increase in the amount of Oskar is not sufficient to rescue the reduction in pole cells in stage 15 embryos. Studying the *P[osk+]/+; Blue*^{2}*/+* embryos, we further found that increasing the amount of *oskar* does not rescue the pole cell phenotype of stage 5 *Blue*^{2}*/+* embryos (Fig 3.5C), and clusters of pole cells are found outside the gonads in stage 15 embryos (Fig 3.5D). These clusters of pole cells in *P[osk+]/+; Blue*^{2}*/+* embryos could be due to defects in pole cell migration (Li, 2003, Santos, 2004b, Van Doren et al., 1998) .

Suppression of the pole cell phenotype by *fpps, columbus* and *quemao* mutants

Normally, the pole cells of wildtype embryos are spherical until they are pulled dorsally along the midgut primordium (PMG) at the onset of gastrulation, when they begin to extend short projections (Brown, 2006, Jaglarz and Howard, 1995). Once the PMG has invaginated, the pole cells extend long projections to migrate through the epithelium of the PMG. The pole cells will then migrate towards the mesoderm, during this time extending and retracting filopodia on their way to the gonad (Brown, 2006, Jaglarz and Howard, 1995, Sano, 2005). In contrast to wildtype, the pole cells of *Blue*$^{-}$ embryos display pseudopodia-like projections as early as the syncytial blastoderm stage. In addition, the pole cells in stage 10 *Blue*$^{-}$ embryos take on a very elongate appearance during migration that is not seen in wildtype embryos (compare Fig 3.4C and E to Figure 3.4A). The early appearance of pseudopodia and the later morphological abnormalities raise the possibility that the mutant pole cells are responding excessively or precociously to a factor that stimulates migration. We therefore asked if inhibition of the pathway proposed to provide the germ cell attractant would alter the *Blue*$^{-}$ phenotype.

Table 3.4 lists the genetic combinations tested for suppression of the pole cell phenotype. Of all the mutants tested, only mutants associated with the isoprenoid biosynthesis pathway - *columbus(hmgcr), fpps, and quemao* - (Santos, 2004b, Van Doren et al., 1998) show a dominant suppression of the pole cell phenotype (Fig 3.6C-E). This suppression occurs maternally, as embryos from *clb*$^{11.5}$*/Blue*^{2}, *fpps*^{K06103}*/+; Blue*^{2}*/+* and *qm*$^{L14.4}$*/Blue*^{2} virgin mothers crossed to w^{1118} fathers show suppression of the phenotype. The final enzyme in the pathway, βGGT1, does not show suppression of the *Blue*$^{-}$ phenotype, however this enzyme may not be as sensitive to reduction, and therefore might not be expected to show suppression dominantly.

Ovaries of *Blue*$^{-}$ females have defects in Staufen and Oskar localization

The reduced numbers of pole cells in stage 5 *Blue*^{2}/+, *Blue*^{2}/*Blue*^{2} or *Blue*^{2}/*Df(3L)ED4543* embryos could be a result of fewer pole cells forming initially. To investigate the idea that fewer pole cells are formed initially, we analyzed the events that occur in oogenesis leading up to pole cell formation. Oskar is the key protein necessary for pole cell formation (Ephrussi and Lehmann, 1992, Lehmann and Nüsslein-Volhard, 1986, Smith et al., 1992), and its mRNA must be properly localized to the posterior of the oocyte during stage 8 in order for translation to occur (Kim-Ha et al., 1991, Kim-Ha et al., 1995). In addition, pole cell formation in the embryo relies on the robust translation of Oskar in a concentrated zone at the posterior pole of the oocyte beginning in stages 9 and 10. This robust translation can only occur if Oskar protein is properly anchored at the posterior (Rongo et al., 1995, Vanzo and Ephrussi, 2002, Webster et al., 1994). In stage 8 wildtype oocytes, Staufen protein, which serves as a marker for *osk* mRNA, is tightly localized to the posterior pole of the oocyte (Fig 3.7A). In *Blue*^{2}/*Blue*^{2} females, though, 32.6% of stage 8 egg chambers have a portion of the Staufen protein mislocalized to the center of the oocyte, while another 39.5% of egg chambers show weaker Staufen staining at the posterior of the oocyte than wildtype (Fig 3.7B and Table 3.5). In stage 10 *Blue*^{2}/*Blue*^{2} oocytes, Staufen is also mislocalized, along with Oskar protein. 15.1% of *Blue*^{2}/*Blue*^{2} egg chambers show a spreading of both Staufen and Oskar proteins away from the posterior cortex (Fig 3.7F, F'), and another 20.8% show weaker staining than wildtype. These phenotypes are not due to gross defects in microtubule polarization in the oocyte, since localization of the microtubule polarity marker Kin:LacZ (Clark et al., 1994) is normal in *Blue*^{2}/*Blue*^{2} egg chambers at both stages (Fig 3.7D, H). This result suggests that the defect in *Blue*$^{-}$ oocytes occurs after microtubule-dependent delivery of *oskar* to the posterior pole (Cha et al., 2002). Even this modest amount of Oskar

mislocalization could account for the reduction in pole cells in stage 5 *Blue^2/Blue^2* embryos, since the formation of pole cells is very sensitive to Oskar concentration at the posterior pole (Vanzo and Ephrussi, 2002, Webster et al., 1994).

In order to determine the localization of the Bluestreak protein in oocytes, we made an antibody to the protein, using the C-terminal 874 aa of the protein as an antigen (see methods). In stage 10 oocytes, Blue is localized to the posterior cortex, as well as in the nuclei of the follicle cells (Fig 3.7I). This staining is largely absent in *Blue^2/Blue^2* egg chambers (Fig 3.7J).

Bluestreak is a novel protein containing NEUR domains

The Bluestreak protein is 1780 amino acids long and contains six NEUR (NHR1) domains and a SPRY domain. The function of both of these domains is still largely unknown. Recent studies on the *neuralized* gene in *Drosophila* (for which the NEUR domain was named), suggest that NHR1 domains mediate protein-protein interactions (Commisso, 2007). Neuralized is an E3 ubiquitin ligase involved in the Notch signaling pathway (Yeh, 2001), and NHR1 domains in Neur are responsible for the interaction between Neur and Delta. The SPRY domain is named after two proteins it was originally identified in, Dictyostelium splA kinase and the rabbit Ca^+ release ryanodine receptor (RyR). The SPRY domain (also known as B30.2 domain) is usually found in one or three copies, and has been identified in nuclear and cytoplasmic proteins, as well as in transmembrane and secreted proteins (Seto, 1999). Structural studies of the SPRY-SOCS box family protein, Gustavus, suggest that SPRY domains also mediate protein-protein interactions (Woo, 2006). Bluestreak is also predicted to have up to eight transmembrane domains, although only 4 of these are above the threshold to be considered "certain" domains (TopPred, Claros, 1994, von Heijne, 1992).

While Bluestreak contains six Neur domains, it is more homologous to mouse mKIAA1787 (36% identity) and human (36% identity) KIAA1787 than *Drosophila* Neuralized (16% identity) or other RING finger containing proteins. The mouse and human KIAA1787 proteins are 95% identical, indicating the extreme conservation of this protein, which is expressed in all tissues at a relatively high level (Nagase, 2001). The identity between Blue and its mammalian counterparts appears to reside only in the Neur domains. Comparing domains individually, the identity between Blue and mouse or human KIAA1787 can be as high as 76%. This is compared with the identity between any of the Neur domains of Bluestreak and either NHR1 domain in Neur, which is only as high as 42%. The C-terminus of the protein contains no conserved domains, however it retains identity with mouse and human KIAA1787 (Nagase, 2001), BLAST). Additionally, Blue does not contain a RING domain, which has previously been shown to be important for the ligase function of Neur (Yeh, 2001). Homologs of Bluestreak not only exist in mouse and humans, but are found in a wide range of animals, including *C. elegans, X. laevis* and *D. rerio*. Interestingly, there are no homologs found in yeast or in plants.

Bluestreak colocalizes with γ-tubulin in the early embryo

In order to determine the localization of the Bluestreak protein in embryos, we took two approaches. First, we made an antibody to the protein (as referred to above). In early embryos, Bluestreak is detected in all cells, and as the embryos cellularize, staining is detected apically in the somatic cells and in two areas per cell in the pole cells (Fig 3.8G). This staining pattern is absent in cells of *Blue*^{2}*/Df(3L)ED4543* embryos (Fig 3.8H). Because the Blue antigen is methanol sensitive, requiring hand-peeling of embryos to obtain a signal (Fig 3.8G), we also monitored protein distribution with a GFP-Blue

reporter, *uas-gfp-Blue,* expressed under the control of the *nos-gal4-vp16* driver. GFP-Blue is detected in a similar localization pattern to α-Blue staining (Fig3.8F, G), localizing to small puncta throughout the embryo, which at cellularization are localized apically (Fig 3.8A-C). Because of this similarity in localization between the two methods of detection, we used *uas-gfp-Blue* embryos in all subsequent experiments. While in somatic cells, GFP-Blue localizes to discrete puncta, the localization of GFP-Blue in pole cells is also occasionally seen spreading away from a central focus (arrow in Fig 3.8C), as well as what appears to be perinuclear localization (Fig3.8D). Owing to the expression pattern of the *nos-gal4-vp16* driver, the GFP-Blue signal is difficult to detect throughout the embryo as gastrulation begins, but the signal persists in pole cells. Even in stage 15 embryos, discrete punctae are still visible, and the perinuclear signal is still apparent (Fig3.8E, arrow).

Since the localization of GFP-Blue was reminiscent of centrosomes, we stained the embryos for γ-Tubulin, which is a component of the centrosomal complex (Raff, 1993). Indeed, throughout early embryonic development, GFP-Blue colocalizes with γ-Tubulin to the centrosomes (Fig 3.8I-L). The localization of GFP-Blue is slightly different as cells in the embryo prepare for cell division, as the GFP-Blue signal is seen to extend away from the distinct focus of γ-tubulin staining at this time (Fig 3.8K and K' and inset).

Centrosomes are still present in *Blue*$^{-}$ mutants

The localization of GFP-Blue to centrosomes raises the question of whether Blue might be an integral component of the centrosome. Considering the phenotype, this is unlikely, since the vast majority of *Blue*$^{-}$ embryos do not show defects in cell division or in patterning, as is observed in mutants for integral components of the centrosome (Li

and Kaufman, 1996). Nevertheless, the integrity of the centrosomes in *Blue^2/Df(3L)ED4543* embryos was evaluated by staining for γ-tubulin. We find that the somatic cells throughout the embryo, and even greatly misshapen pole cells, still contain punctate γ-tubulin staining (Fig 3.9B, arrows), suggesting that Bluestreak is not an integral component of the centrosome.

DISCUSSION

Here we present that the novel protein Bluestreak plays a role in proper pole cell behavior during migration in the *Drosophila* embryo. We have shown that at stage 5, *Blue$^-$* embryos display defects in pole cell morphology, a phenotype that closely resembles migrating pole cells in shape. We have further shown that these defects in shape can be suppressed by mutations in the isoprenoid biosynthesis pathway, which has been implicated in the provision of guidance cues for pole cell migration. Finally, we have demonstrated that Bluestreak colocalizes with γ-tubulin at the centrosomes throughout the embryo and also localizes around the nucleus in pole cells. While it is still not clear which fraction of Blue localization is important for the functions characterized here, we discuss possible scenarios below. Overall, we conclude that Bluestreak functions to suppress migratory behavior in the pole cells of early embryos, and may continue to act as a regulator of guided migration as the embryo matures.

Possible model for Bluestreak function

We have shown that mutations in the isoprenoid biosynthesis pathway suppress the *Blue$^-$* pole cell phenotype of early embryos. Isoprenoid biosynthesis has been shown to affect the ultimate destination of the pole cells, since mutants for enzymes in the

pathway lead to mismigrated pole cells that do not reach the gonad. Santos and Lehmann (2004b) suggest that the pathway works to produce a "germ cell attractant", since it is possible to attract the pole cells to ectopic locations through misexpression of these enzymes. These experiments were performed using stage 10-15 embryos, and demonstrate that the expression pattern of these enzymes is critical in determining the path of migration of the pole cells. Since these enzymes are also loaded into the early embryo from maternal stores (BDGP), they have the potential to affect the migratory behavior of early pole cells as well. Despite the broad expression of these enzymes in wildtype early embryos, the pole cells remain spherical and do not attempt to migrate at this stage. There are two possible explanations for this lack of migratory response in the pole cells of wildtype early embryos. First, either the pole cells do not have the machinery to respond to the attractant, or second, there is a mechanism to prevent the response to the attractant in early embryos. We contend that there is a mechanism to prevent the response of the pole cells to the attractant in early embryos and that this mechanism involves Bluestreak. Figure 3.10 depicts the possible role that Bluestreak plays to prevent early migration. In the wildtype situation (Fig 3.10B), the transcripts for *columbus, fpps* and *quemao* are all present in the early embryo, yet the pole cells are spherical due to suppressive action of Bluestreak. Reducing the amount of Bluestreak present in early embryos lifts this suppression and the pole cells begin to show migratory behavior (Fig 3.10C). However, if the amount of Bluestreak and either *columbus, fpps* or *quemao* supplied by the mother to the embryo is reduced (Fig 3.10D), then the pole cells again appear spherical. This result suggests that reducing the amount of *columbus, fpps* or *quemao* leads to a reduction in the amount of attractant produced and therefore a reduction in the migratory response of the pole cells in early embryos. Without this

attractive signal, the pole cells do not attempt to migrate and the need for Bluestreak to suppress migratory behavior in early embryos is abrogated.

The interaction of Bluestreak with other controls of germ cell migration

While the *Blue*$^{-}$ phenotype is comparable to gain-of-function mutations in the torso pathway, in which the pole cells are seen migrating through the blastoderm embryo (Li, 2003), we have so far not been able to implicate Blue as a factor in this pathway. Embryos from mothers homozygous for a strong allele of *tor*, *tor*^{XR1}, have pole cells which are immotile, even in culture, with few if any indications of pseudopodia (Li, 2003). We tested to see if *Blue*^{2} could suppress this non-motile phenotype, however embryos from *tor*^{XR1}/*tor*^{XR1}; *Blue*^{2}/*Blue*^{2} mothers are identical to embryos from *tor*^{XR1}/*tor*^{XR1} mothers. The pole cells in embryos from mothers of either genotype remain outside the embryo, even by stage 15 (Li, 2003). It is possible that *torso* is epistatic to *Blue* and therefore either genotype shows the *torso* phenotype. We are currently testing whether the mutants in *Blue* can enhance the gain-of-function phenotype of *tor*^{Y9} mutants, and also whether *qm*$^{L14.4}$ and *clb*$^{11.5}$ can suppress the *tor*^{Y9} gain-of-function phenotype. Furthermore, torso signaling has also been shown to affect the migration of the border cells in the egg chamber (Silver and Montell, 2001). We looked at the effects of mutations in *Blue* on border cell migration and found no differences from wildtype, indicating that Blue does not act in this process.

Implications of the localization of Bluestreak in embryos

The specific way in which Bluestreak acts to suppress pole cell migration in early embryos is still unknown. In general, it appears that the suppression is temporal and is relieved at the developmentally appropriate time in response to guidance cues. Since we

find that Blue is localized to both the nuclear membrane and pericentrosomally in pole cells, and not to the plasma membrane, we believe that Blue responds to these cues indirectly. The way in which Blue could act at the nuclear membrane to mediate a response to the germ cell attractant is difficult to speculate at this point with the results we have obtained. We can imagine, though, that Bluestreak could be an integral nuclear membrane component, since it contains a SPRY domain and is predicted to have up to eight transmembrane domains. The localization of Blue to centrosomes offers two alternative ways in which Blue might mediate the response to the germ cell attractant. Riparbelli et al (2007) have shown that disruption of the centrosomes in early embryos also disrupts cortical microfilament restructuring. Considering this, Blue could be acting at the interface between centrosomes and the actin cytoskeleton, perhaps acting as a cofactor that suppresses the migratory response until the appropriate time. On the other hand, the localization of Bluestreak around the centrosomes may reflect an association with endosomes. A localization pattern similar to Blue has been detected for Rab11, a small GTPase which colocalizes with γ-tubulin to endosomal compartments (the subapical compartment (SAC) in particular) and is involved in endosomal trafficking (Pelissier et al., 2003). Endosomal trafficking has been implicated in the regulation of cell migration, since membrane proteins and receptors important for migration are recycled to the leading edge of the migrating cell through endosomes (Jones et al., 2006, and references therein). The localization of Blue around centrosomes (the location of the SAC) opens the possibility that Blue could act to regulate migration through endosomal recycling. Perhaps the attractant signal is internalized in endosomes, and Blue acts to recycle internalized attractant back outside the cell until the appropriate time. In addition, the fact that Blue contains NHR1 domains also suggests that Blue could act in the internalization of molecules. The NHR1 domains of Neuralized have been shown to be

important for the endosomal internalization of Delta (Commisso and Boulianne, 2007). Mutation of a highly conserved glycine (G167E) residue in the first of the NHR1 domains in Neuralized disrupts Delta internalization. This invariant glycine is also present in NHR1 domains 2, 3 and 4 in Blue, consistent with the idea that these domains may act similarly in Bluestreak for internalization of molecules.

Implications of the localization of Bluestreak in oocytes

The connection between the centrosomes and microfilaments via Blue, as well as a role for Blue in endosomal trafficking, are each attractive models considering the mislocalization of Staufen and Oskar proteins in $Blue^2/Blue^2$ stage 10 oocytes. As a link between the centrosomes and actin, Blue may directly affect the actin-dependent localization of *osk*. Cha et al. (2002) showed that disruption of the actin cytoskeleton in stage 10 oocytes using cytochalasin D leads to a dissociation of *osk* mRNA from the posterior of the embryo. Furthermore, mutants for the actin binding proteins Moesin and Tropomyosin have been also been shown to disrupt the posterior anchoring of Osk in the oocyte (Erdélyl et al., 1995, Jankovics et al., 2002, Polesello et al., 2002, Tetzlaff, 1996). We find a similar mislocalization of Osk in $Blue^2/Blue^2$ stage 10 oocytes, suggesting that the organization of actin at the posterior may be perturbed. Then again, the localization of Bluestreak to the posterior pole of the oocyte could reveal a connection for Blue with recycling endosomes in the oocyte. The endosomal component Rab11 is also localized to the posterior pole of the oocyte and contributes to *osk* mRNA localization and translation (Dollar et al., 2002). Furthermore, it has been shown that the long isoform of Osk protein itself localizes to these endosomes at the posterior pole and thereby reinforces its own localization to the posterior (Vanzo et al., 2007). Blue could also act as a component of these endosomes to maintain the posterior localization of *osk* mRNA and Osk protein,

allowing for efficient accumulation of the translated Osk product. If Bluestreak is involved in endosomal trafficking in the oocyte, it acts less generally than Rab11, since processes such as microtubule plus end focusing in the oocyte appear normal in *Blue*$^{-}$ oocytes.

MATERIALS AND METHODS

Fly stocks and transgenes

Df(3L)fz-GF3b, *Df(3L)ED4543, Df(3L)Exel6120, Df(3L)Exel6121, P[EY12221] (Blue*^{1}*), P[hs-hid]* and *fpps*^{K06103} were obtained from the Bloomington Stock Center. *Df(3L)Blue*^{19b} was made using stocks F01830 and D06126 from the Bloomington Stock Center and the method of (Parks et al., 2004). *Blue*^{2} and *Blue*^{3} were made using standard excision methods. Single fly PCR from *Blue*^{2}*/Df(3L)Blue*^{19b} and *Blue*^{3}*/Df(3L)Blue*^{19b} flies was used to sequence the alleles at the UT ICMB Core Facility. Precise excision of the *P[EY12221]* element leads to a loss of the phenotype in embryos. Mutants in *tre1*$^{\Delta EP5}$, *clb*$^{11.5}$, *qm*$^{L14.4}$, *βggt*^{xs2554} and *wun*^{ce} were kindly provided by the Lehmann Lab. Kin:LacZ flies were provided by the Stein Lab.

BAC clone RP35M1 was obtained from the RPCI-98 Drosophila melanogaster BAC Library of the Children's Hospital Oakland Research Institute. RP35M1 was digested with XbaI and NotI to obtain an 11.5 Kb fragment which would include the *Blue* locus and this was confirmed by PCR. *P[Blue*$^{+}$*]* is a genomic fragment of this subclone containing the *Bluestreak* transcription unit as well as 1.6 Kb upstream of the start codon and .4 Kb downstream of the poly A site. To insure that *P[Blue*$^{+}$*]* did not contain portions of the nearby CG68333 gene, primers GGGGTACCCACCGACCAGTGTGACCGTC and GCGGCCACAGCGTGTGTGG were used to synthesize 1.4 Kb fragment introducing an

Asp718 site to the 5' end. The 8.0 Kb Asp718 to NotI fragment was inserted into the pCaSpeR vector for P element transformation (Pirotta, 1988) modified with Asp718 and NotI sites in the PstI and EcoRI sites, respectively.

P[uas-gfp-Blue] was constructed using primers GAAGATCTATGACCGG-CAGAGAAGCGC and GGAACCACTTCCATCGCGCG to synthesize a .5 kb fragment containing an added BglII site directly before the start codon. An XbaI linker was put into a NotI site that is .6 kb after the stop codon. The entire 6.4 kb BglII to XbaI fragment was then ligated into pUASg (Pernille Rorth (Rorth, 1998)) containing mGFP6 from Andrea Brand. The construct was driven using a nos-gal4-vp16 driver.

Antibody production and staining

SD03524 is a partial cDNA for *Bluestreak* obtained from the Drosophila Genomics Resource Center. It encodes for the C-terminal 874 amino acids of the protein. This cDNA was inserted into the pET15b expression vector using NarI and XhoI sites, and the protein was expressed in pLysS. The partial protein was purified by insoluble aggregate purification and sent to ProteinTech for antibody production. The antibody was affinity purified using the initial antigen (~80kD) transferred onto nitrocellulose strips.

Embryos were collected stained as described previously ((Macdonald et al., 1986, Macdonald et al., 1991), except for *P[hs-hid]* collection, and staining with α-Blue. For *P[hs-hid]* collection, embryos were collected in apple juice vials for one hour, and then heat shocked for one hour at 37ºC. After one hour of recovery, the embryos were processed as usual. For α-Blue staining, since the Blue antigen is methanol sensitive, embryos were hand-peeled to remove the vitelline membrane, and were then processed as usual. Antibodies were used at the following concentrations: rabbit α–Blue, 1:200; mouse-α–γ–tubulin (GTU-88, Sigma-Aldrich), 1:500; rabbit α-Caspase-3, 1:500; rat α-

Vasa, 1:500; rat α-Staufen, 1:1000; rabbit α-Oskar, 1:3000; mouse α-LacZ, 1:100. Cy5 and Alexa-fluor 488 (Jackson Immunoresearch Laboratories) were used 1:800, Cy3 donkey α-mouse was used at 1:150, Topro-3 was used at 1:1000 (Molecular probes). Stained embryos were mounted in Vectashield medium (Vector Labs) and imaged using a Leica TCS-SP confocal microscope.

TABLES

Table 3.1: Percentage of stage 5 embryos from mothers of the given genotype that exhibit the pole cell phenotype.

Genotype	Wildtype Pole cells	Misshapen Pole cells	n
w^{1118}	100.00%	0	90
Df(3L)fz-GF3b/+	31.2	68.8	96
Df(3L)ED4543/+	19.2	80.8	78
Df(3L)Exel^{19b}/+	37.3	62.7	67
Blue^{1}/+	89.3	10.7	103
Blue^{1}/Blue^{1}	85.5	14.5	138
Blue^{2}/+	20.7	79.3	87
Blue^{2}/Blue^{2}	12.5	87.5	56
Blue^{2}/Df(3L)ED4543	10.6	89.4	47
P[Blue^{+}]/+; Blue^{2}/+	100.0	0.0	52
Blue^{3}/+	11.8	88.2	51

Table 3.2: Adult daughters of *Blue*$^{-}$ mothers can be agametic.

Genotype of mother	Two ovaries	One ovary	No ovaries	n
Df(3L)fz-GF3b/+	78.3%	21.7	0.0	23
Blue^{2}*/+*	100.0	0.0	0.0	22
Blue^{2}*/Blue*^{2}	78.9	15.8	5.3	19
Blue^{2}*/Df(3L)fz-GF3b*	66.7	22.2	11.1	18

Table 3.3: *Blue*$^{-}$ embryos have reduced pole cell number.

Genotype	Pole cell # Stage 5 (per embryo)	n	Pole cell # Stage 15 (per gonad)	n
w^{1118}	36.2	25	15.5 ± 1.7	16
Blue^{2}*/+*	25.8	24	8.9 ± 4.3	26
Blue^{2}*/Blue*^{2}	15.8	12	6.1 ± 4.0	34
Blue^{2}*/Df(3L)ED4543*	9.4	17	4.3 ± 2.3	24
P[Blue^{+}*]/+; Blue*^{2}*/+*	34.6	17	10.8 ± 2.9	20
P[osk^{+}*]/+; Blue*^{2}*/+*	49.6	14	12.5 ± 2.8	22
P[osk^{+}*]/+*	51.5	28	15.8 ± 3.4	20

Table 3.4: Maternal mutations in *columbus*, *quemao* and *fpps* dominantly suppress the pole cell phenotype of *Blue*$^{-}$ embryos.

Genotype of mother*	Average pole cell number Stage 5	% of embryos with misshapen pole cells	n
Blue^{2}/+	26.4	73.7	76
clb$^{11.5}$/ *Blue*^{2}	29.4	15.4	26
qm$^{L14.4}$/ *Blue*^{2}	25.9	14.3	35
fpps^{K06103}/+; *Blue*^{2}/+	30.6	19.6	51
βGGT^{xs2554}/+; *Blue*^{2}/+	27.3	62.1	29
wun^{ce}/+; *Blue*^{2}/+§	nd	73.9	23
tre-1$^{\Delta EP5}$/+; ; *Blue*^{2}/+§	nd	76.5	34

*All mothers were virgin females crossed to *w*^{1118} males, except those marked with §, which were crossed to males of the same genotype as the females.

nd - not determined

Table 3.5: Ovaries of *Blue*$^{-}$ females have Staufen and Oskar localization defects.

Genotype	Staufen localization, Stage 8			n
	Wildtype	Mislocalized		
		Weak	Dot	
W1118	100%	0.0	0.0	45
Df(3L)fz-GF3b/+	70.5	29.5	0.0	44
Df(3L)Exel19b/+	37.3	43.1	19.6	51
Blue^{2}/+	52.0	18.8	29.2	48
Blue^{2}/*Blue*^{2}	27.9	39.5	32.6	43

Genotype	Staufen/Oskar localization Stage 10			n
	Wildtype	Mislocalized		
		Weak	Spread	
W1118	100%	0.0	0.0	54
Df(3L)fz-GF3b/+	68.4	31.6	0.0	57
Df(3L)Exel19b/+	58.8	25.5	15.7	51
Blue^{2}/+	57.1	42.9	0.0	49
Blue^{2}/*Blue*^{2}	64.1	20.8	15.1	53

FIGURES

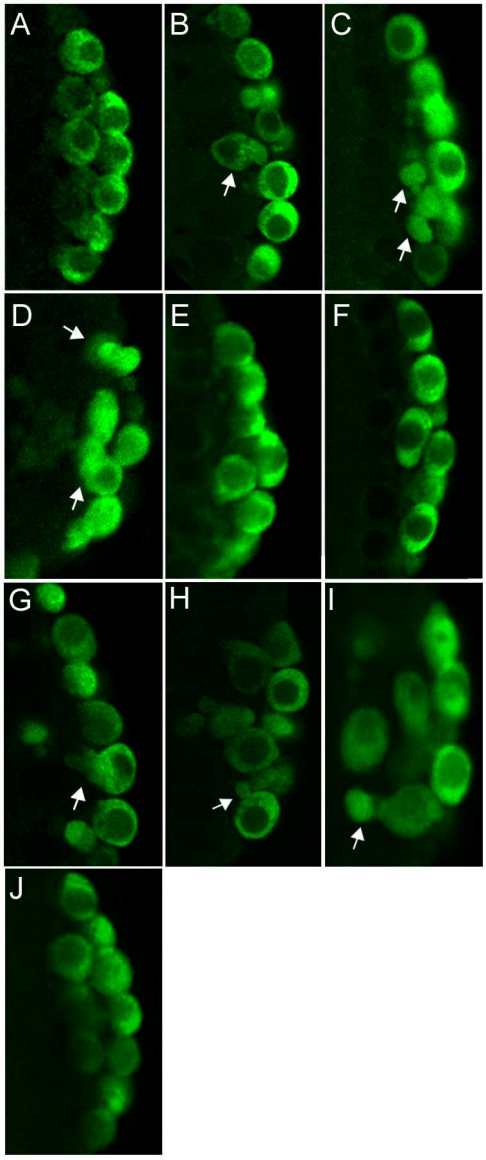


Figure 3.1: Reducing the amount of *Bluestreak* in mothers results in an embryonic pole cell phenotype.

All embryos were stained with α-Vasa (green) to detect pole cells. A-F. Embryos from mothers which are deficient for *Blue* display a distinctive pole cell phenotype. The pole cells of embryos from wildtype mothers (A) are spherical and regular in size, while the pole cells of embryos from *Df(3L)fz-GF3b/+* (B), *Df(3L)ED4543/+* (C), and *Df(3L)Blue*^{19b}*/+* (D) show defects in both size and shape (arrows indicate examples of misshapen cells). It is important to note that this phenotype is dominant. In contrast, deficiencies which do not delete *Blue*, *Df(3L)Exel6120* (E) and *Df(3L)Exel6121* (F), do not show the phenotype.

G-J. Mutants in *Blue* display the same distinctive pole cell phenotype. Pole cells of embryos from *Blue*^{1}*/+* (G), *Blue*^{2}*/+* (H) and *Blue*^{3}*/+* (I) mothers are also misshapen (arrows). In contrast, the pole cells of embryos from *P[Blue*$^{+}$*]/+; Blue*^{2}*/+* mothers (J), do not show the phenotype, demonstrating that one copy of a genomic construct including *Blue* is capable of rescuing the pole cell phenotype of *Blue*^{2} embryos.

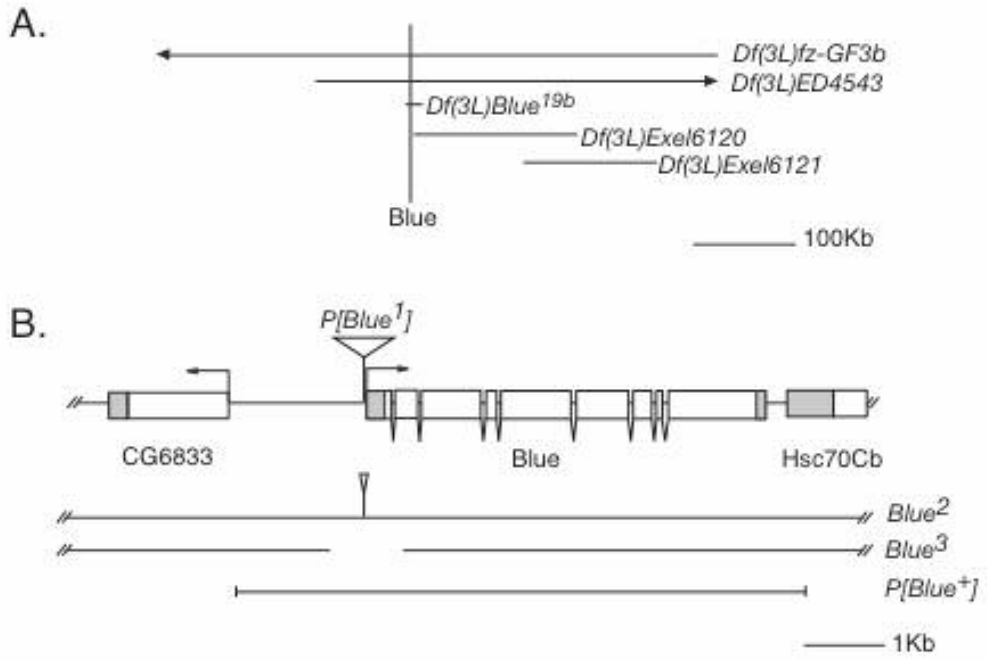


Figure 3.2: Deficiencies deleting *Bluestreak* and the gene model for the region.

A. Depiction of the position of *Bluestreak* in relationship to several deficiencies in the region. Deficiencies *Df(3L)fz-GF3b*, *Df(3L)ED4543*, and *Df(3L)Blue^{19b}* (see methods) delete *Blue*, while *Df(3L)Exel6120* and *Df(3L)Exel6121* do not. B. The *Blue* locus is closely flanked by *CG6833* upstream, and *Hsc70Cb* downstream. Both *CG6833* and *Hsc70Cb* are transcribed in the opposite direction as *Blue*. The *Blue^{1}* insertion resides 4 bp before the predicted transcription start site for *Blue*. In *Blue^{2}*, a small portion of the P-element remains, while in *Blue^{3}*, the first exon and a portion of the second exon are deleted, as well as 409 bp upstream of the transcription start site, as indicated by the break in the line. The genomic rescue construct, *P[Blue^{+}]*, contains the region depicted by the lowest line.

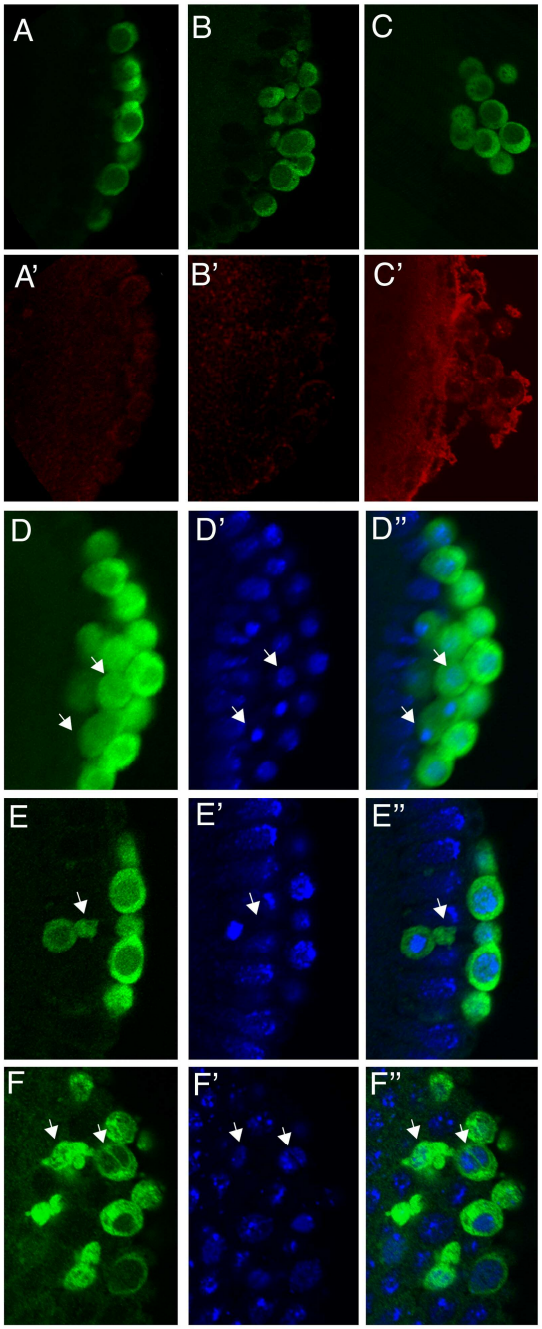


Figure 3.3: Misshapen pole cells are not Caspase-3 positive and have normal nuclear morphology.

A-C. Paired panels (e.g. A and A') show Vasa (green) and Caspase-3 (red) staining of wildtype (A, A'), *Blue*^{2}/+ (B, B'), and *P[hs-hid]/+* (C,C') embryos, all of which have been heat shocked for one hour. Only the *P[hs-hid]/+* embryos show Caspase-3 staining in the pole cells, suggesting that the *Blue*^{2}/+ embryos are not dying via the Caspase induced apoptosis pathway. D-F. Each row shows Vasa staining (green), DNA visualized with Topro-3 (blue) and merge for the following genotypes: wildtype (D, D', D''), *Blue*^{2}/+ (E, E', E''), *Blue*^{2}*/Df(3L)ED4543* (F, F', F''). In pole cells of wildtype embryos, the nuclei are large and round, even in dividing cells (arrows). In the misshapen pole cells of *Blue*^{2}/+ embryos, the smaller tag attached to the cell does not contain DNA (arrow), however the nucleus in the main body of the cell appears normal. This is true for all misshapen pole cells of this genotype. However in some *Blue*^{2}*/Df(3L)ED4543* embryos, the occasional pole cell contains two nuclei (arrow). Note

Figure 3.3: Misshapen pole cells are not Caspase-3 positive and have normal nuclear morphology (contd.).

that the other misshapen pole cells in the panel only have one nucleus despite the contorted shape of the cells.

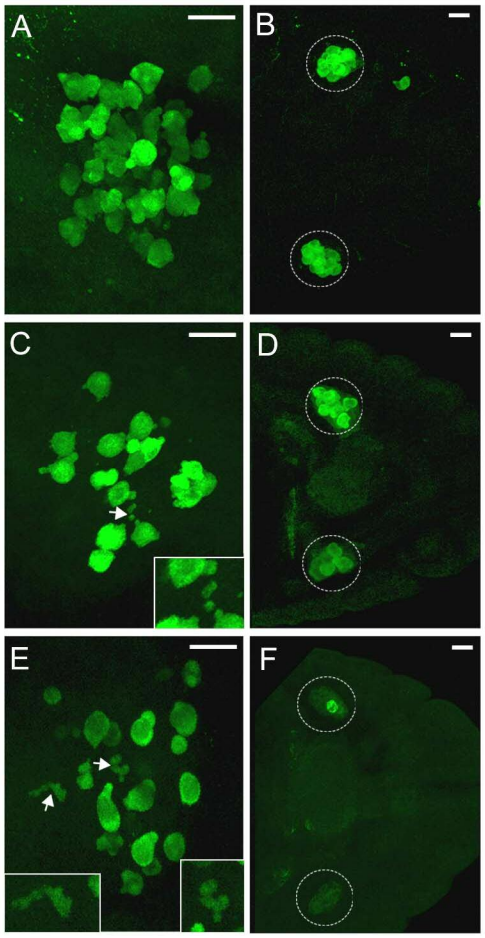


Figure 3.4: Pole cells of *Blue*$^{-}$ embryos also show defects during migration and a reduction in the number that reach the gonad.

All panels show stereo projections of a series of confocal sections to display all of the pole cells in each embryo. Embryos were stained with α-Vasa (green) to detect pole cells. Panels A, C and E show embryos at stage 10, when the pole cells are migrating out of the midgut primordium. While in wildtype (A) the pole cells show small projections, *Blue*^{2}/+ (C) or *Blue*^{2}/ *Blue*^{2} (E) embryos have pole cells that are greatly elongated and considerably misshapen (arrows, and insets in C and E). Panels B, D and F show embryos at stage 15, when the pole cells have coalesced with the somatic cells of the gonad. In wildtype embryos (B), an average of 15.5 pole cells are found in each gonad (circles). However in *Blue*^{2}/+ (D) or *Blue*^{2}/ *Blue*^{2} (F) embryos, the number of pole cells in the gonads is reduced, so that in some *Blue*^{2}/ *Blue*^{2} embryos, there are few or no pole cells in found in the gonads (circles). Scale bars in all panels are 20 μm.

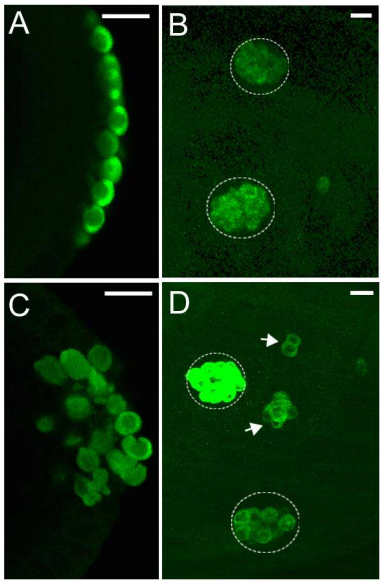


Figure 3.5: *P[osk^{+}]/+; Blue^{2}/+* embryos have increased pole cell number, but still show the misshapen pole cell phenotype.

All panels show embryos stained with α-Vasa (green) to visualize the pole cells. A and C. Stage 5 *P[osk+]/+* embryos (A) have increased numbers of pole cells, yet they are spherical as in wildtype (Fig 3.1A). The pole cells of *P[osk^{+}]/+; Blue^{2}/+* embryos are still misshapen, even though the number of pole cells is increased. B and D. The pole cells of stage 15 *P[osk+]/+* embryos (B) are found in the gonads (circles), with an occasional pole cell outside this region, similar to wildtype embryos (Fig 3.4B). On the other hand, in stage 15 *P[osk^{+}]/+; Blue^{2}/+* embryos, pole cells are found in clusters (arrows) outside the region of the SGP's (circles). Scale bars in all panels are 20 μm.

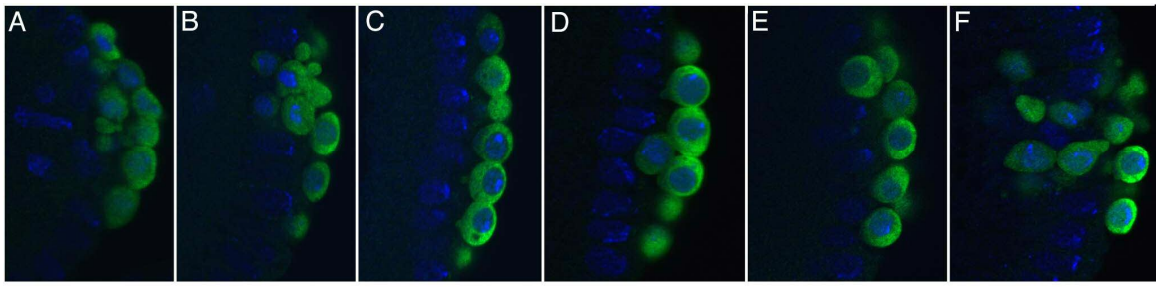


Figure 3.6: Maternal mutations in *columbus*, *quemao* and *fpps* dominantly suppress the pole cell phenotype of embryos from *Blue*$^{-}$ mothers.

A-F. All panels show α-Vasa staining (green) of representative embryos from virgin females of a given genotype. Embryos from *tre-1*$^{\Delta EP5}$*/+;+/+; Blue*^{2}*/+* (A) *wun*^{ce}*/+; Blue*^{2}*/+* (B) and *βGGT*$^{xs\text{-}2554}$*/+; Blue*^{2}*/+* (F) mothers still show the misshapen pole cell phenotype of embryos from *Blue*^{2}*/+* mothers. In contrast, the vast majority of embryos from *clb*$^{11.5}$*/Blue*^{2} (C), *fpps*^{K06103}*/+; Blue*^{2}*/+* (D) and *qm*$^{L14.4}$*/Blue*^{2} (E) mothers have pole cells of normal morphology.

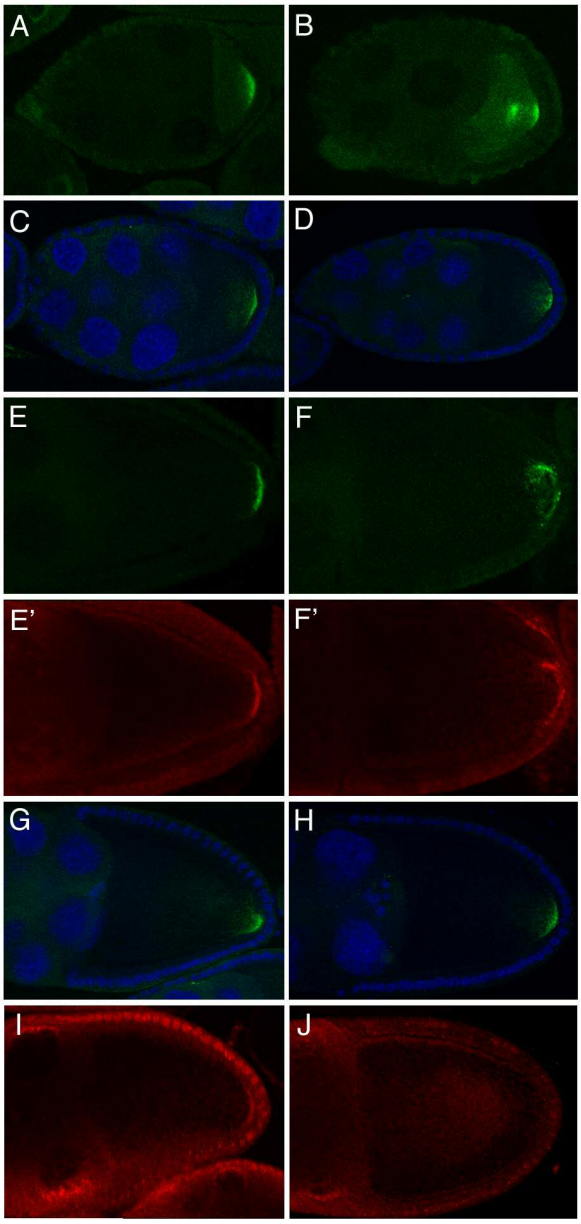


Figure 3.7: Ovaries of *Blue*$^{-}$ females have Staufen and Oskar localization defects, despite proper Kin-LacZ localization.

A. and B. Stage 8 ovaries stained with α-Staufen (green). In wildtype ovaries (A), Staufen is tightly localized to the posterior pole, while in *Blue*^{2}/ *Blue*^{2} ovaries (B), Staufen protein is frequently mislocalized to the center of the oocyte. C. and D. The localization of the microtubule polarity marker, Kin:LacZ (green), is localized to the posterior pole in both wildtype (C) and in *Blue*^{2}/ *Blue*^{2} (D) ovaries, indicating that microtubule organization is largely normal in the mutant. DNA (blue) is visualized using Topro-3. E-F. Paired panels show Staufen (green) and Oskar (red) localization in wildtype (E and E') and in *Blue*^{2}/ *Blue*^{2} (F and F') stage 10 ovaries. While both Staufen and Oskar are tightly localized to the posterior in wildtype (E and E') ovaries, both proteins are seen falling away from the posterior in some *Blue*^{2}/ *Blue*^{2} (F and F') ovaries. Panels G and H show that Kin:LacZ localization is similar in stage 10 ovaries of both wildtype (G) and in *Blue*^{2}/ *Blue*^{2} (H) females. I. and J. α-Blue staining of wildtype and *Blue*^{2}/*Blue*^{2} stage 10 egg chambers. Blue is localized to the posterior cortex

Figure 3.7: Ovaries of *Blue*$^{-}$ females have Staufen and Oskar localization defects, despite proper Kin-LacZ localization (contd.).

of the oocyte and in the nuclei of follicle cells in stage 10 wildtype egg chambers (I), this staining is largely absent in *Blue*^{2}*/Blue*^{2} stage 10 egg chambers (J).

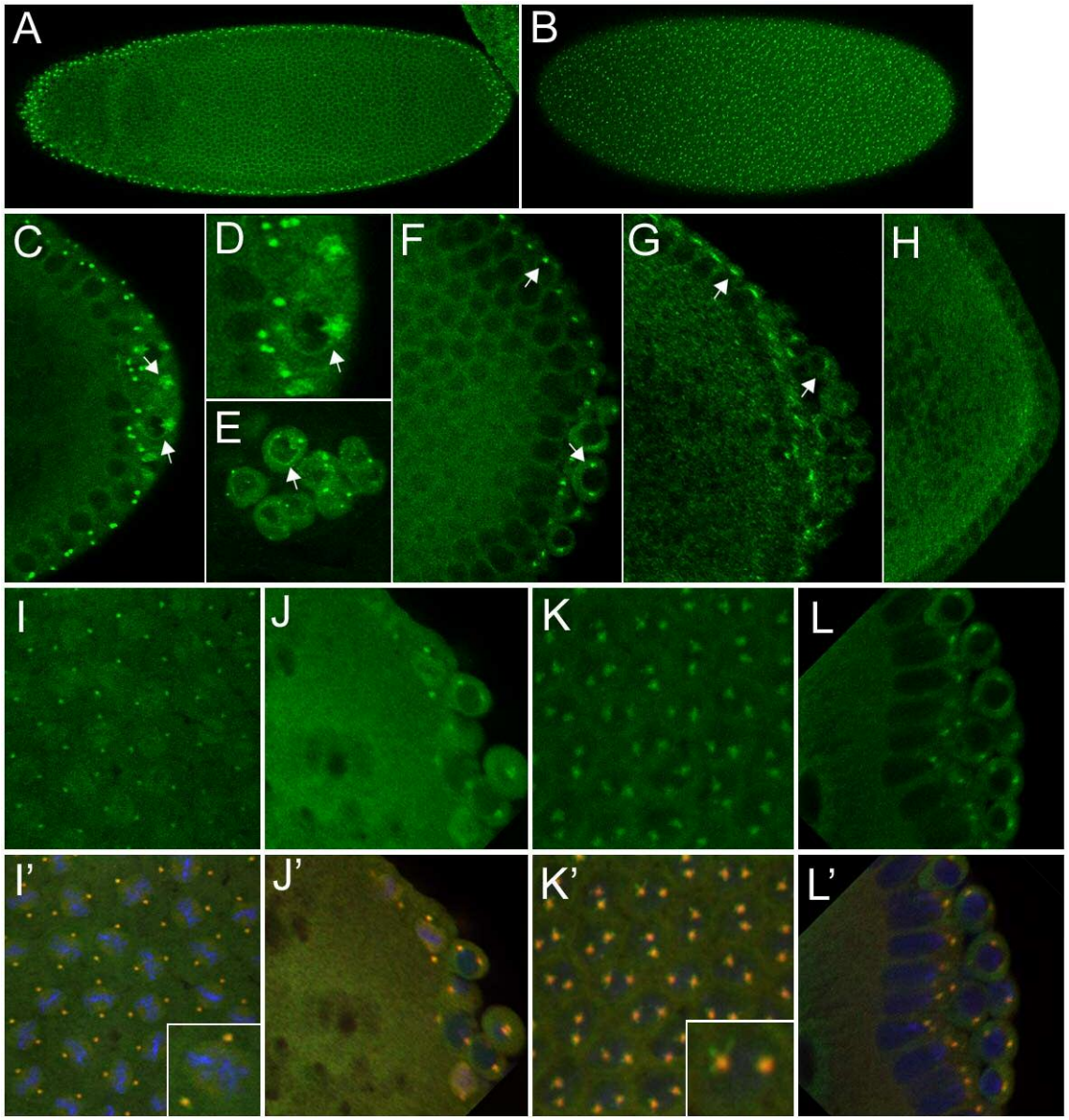


Figure 3.8: Subcellular localization of GFP-Blue in embryos from *P[uas-gfp-Blue]/P[nos-gal4-vp16]* mothers is similar to α-Blue staining, and colocalizes with γ-tubulin.

A-B. GFP-Blue (green) is localized throughout the embryo in two puncta per cell which are oriented apically (A). A confocal section of the surface of the embryo (B). C-E. In the

Figure 3.8: Subcellular localization of GFP-Blue in embryos from *P[uas-gfp-Blue]/P[nos-gal4-vp16]* mothers is similar to α-Blue staining, and colocalizes with γ-tubulin (contd.).

pole cells of a syncytial blastoderm embryo (C), GFP-Blue also spreads just beyond the two foci in each cell, and appears to localize perinuclearly (arrows). This localization is shown in more detail in D (arrow), and is also seen in the pole cells of stage 15 embryos (E, arrow). F-G. The localization of GFP-Blue (F) is very similar to the staining detected using α-Blue antibodies (G). Arrows point to the puncta seen with either detection method. H. α-Blue staining is largely absent in embryos from *Blue^{2}/Df(3L)ED4543* mothers. I-L. Paired panels show the localization of GFP-Blue (green, I-L) and merge of GFP-Blue (green), γ-tubulin (red) and Topro-3 (blue) staining (I'-L'). I and I', somatic cells of the syncytial blastoderm embryo, showing GFP-Blue localized to very small puncta, and perfectly colocalized with γ-tubulin (see inset); J and J', pole cells of the syncytial blastoderm embryo showing similar localization and colocalization; K and K', somatic cells of the cellular blastoderm embryo, showing how the GFP-Blue signal is sometimes extending away from the γ-tubulin signal (see inset); L and L', pole cells of the cellular blastoderm embryo.

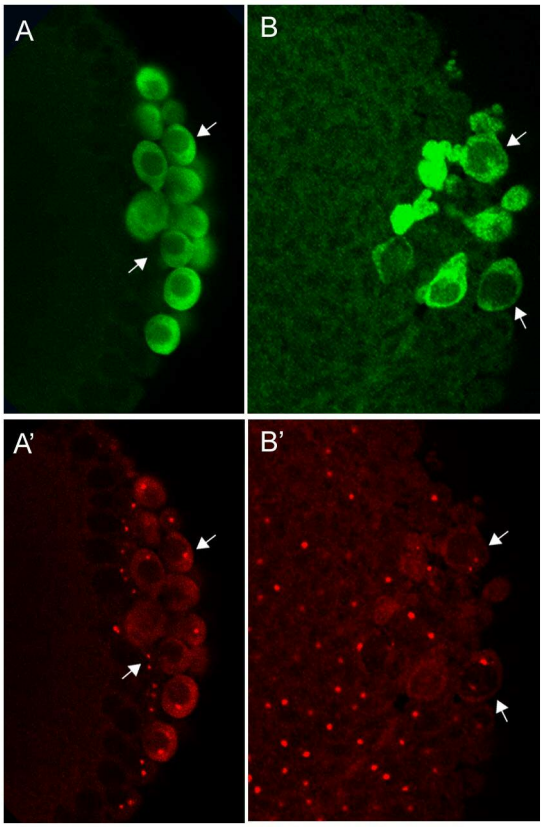


Figure 3.9: Centrosomes are still present in *Blue*$^{-}$ embryos.

Panels A and B show Vasa (green) staining to identify pole cells and panels A' and B' show γ–tubulin staining (red) to visualize the centrosomes. Paired panels depict different confocal planes within each embryo to enhance detection of the centrosomes in the pole cells. In wildtype embryos (A and A'), centrosomes are easily detectable as small puncta in both the somatic cells and in the pole cells (arrows). In *Blue*^{2}*/Df(3L)4543* embryos (B and B'), centrosomes are still detected even in misshapen pole cells (arrows). Note also that centrosomes are still intact in the somatic cells.

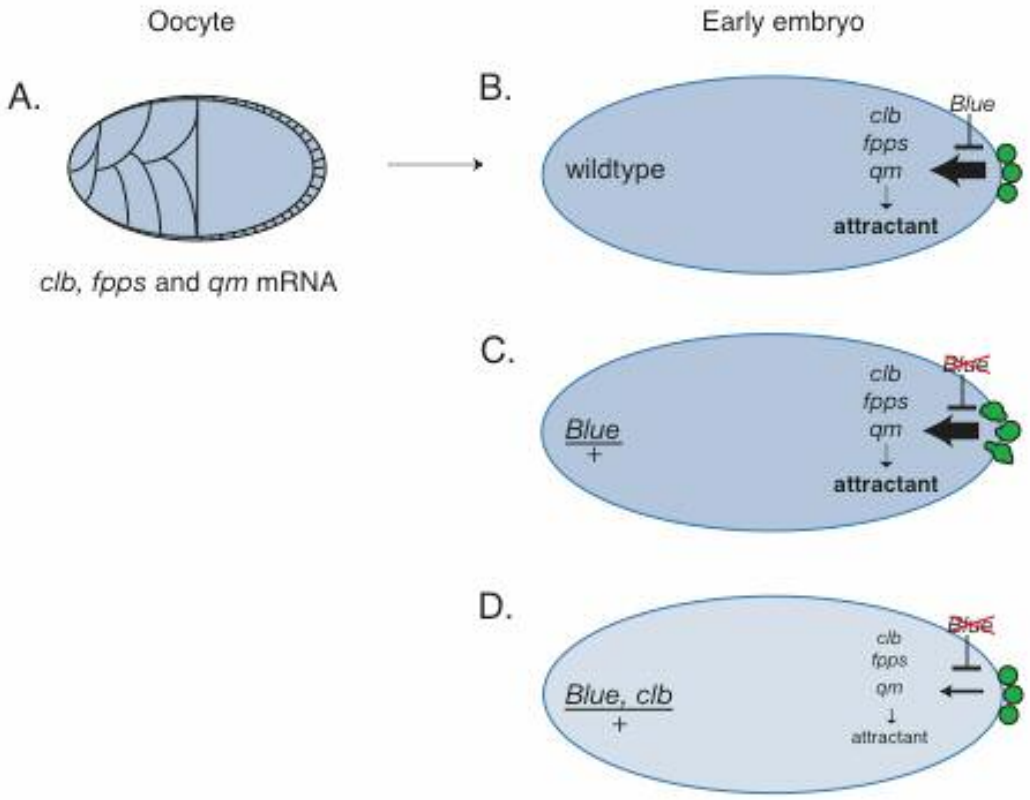


Figure 3.10: Model for Bluestreak function.

The transcripts for *clb, fpps* and *qm* are produced by the nurse cells and loaded into the oocyte (A) and deposited in the early embryo (B). This maternal contribution has the potential to produce the germ cell attractant and cause the pole cells to exhibit migratory behavior (i.e. extend pseudopodia) in early embryos. Yet the pole cells remain spherical in wildtype, presumably because Blue acts to prevent this attraction. In the case where Blue protein is reduced (C), the pole cells indeed show migratory behavior, extending pseudopodia. This phenotype can be suppressed if the expression level of *clb, fpps,* or *qm* is reduced (D). These data indicate that the pole cells are competent for migration and can show signs of migratory behavior in the presence of the attractant, yet migration is suppressed in wildtype by the action of Bluestreak.

REFERENCES

Bellen, H. J., R. W. Levis, G. Liao, Y. He, J. W. Carlson, G. Tsang, M. Evans-Holm, P. R. Hiesinger, K. L. Schulze, G. M. Rubin, R. A. Hoskins, and A. C. Spradling. 2004. The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics.* 167:761-781.

Breitwieser, W., F.-H. Markussen, H. Horstmann, and A. Ephrussi. 1996. Oskar protein interaction with vasa represents an essential step in polar granule assembly. *Genes Dev.* 10:2179-2188.

Brennecke, J., D. R. Hipfner, A. Stark, R. B. Russell, and S. M. Cohen. 2003. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell.* 113:25-36.

Brown, S., Zeidler, M.P., Hombria, J.E. 2006. JAK/STAT signaling in Drosophila controls cell motility during germ cell migration. *Dev Dyn.* 235:958-966.

Callaini, G., Riparbelli, M.G., Dallai, R. 1995. Pole cell migration through the gut wall of the Drosophila embryo: analysis of cell interactions. *Dev Biol.* 170:365-375.

Campos-Ortega, J. A. a. H., Volker. 1985. The Embryonic Development of Drosophila melanogaster.

Cha, B. J., L. R. Serbus, B. S. Koppetsch, and W. E. Theurkauf. 2002. Kinesin I-dependent cortical exclusion restricts pole plasm to the oocyte posterior. *Nat Cell Biol.* 4:592-598.

Clark, I., E. Giniger, H. Ruohola-Baker, L. Y. Jan, and Y. N. Jan. 1994. Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr. Biol.* 4:289-300.

Claros, M. G. a. v. H., G. 1994. TopPred II: An improved software for membrane protein structure predictions. *CABIOS.* 10:685-686.

Coffman, C. R., Strohm, R.C., Oakley, F.D., Yamada, Y., Przychodzin, D., Boswell, R.E. 2002. Identification of X-linked genes required for migration and programmed cell death of Drosophila melanogaster germ cells. *Genetics.* 126:273-284.

Commisso, C., Boulianne, G.L. 2007. The NHR1 domain of Neuralized binds Delta and mediates Delta trafficking and Notch signaling. *Mol Biol Cell.* 18:1-13.

Deshpande, G., Swanhart, L., Chiang, P., Schedl, P. 2001. Hedgehog signalling in germ cell migration. *Cell.* 106:759-769.

Dollar, G., Struckoff, E., Michaud, J., Cohen, R.S. 2002. Rab11 polarization of the *Drosophila* oocyte: a novel link between membrane trafficking, microtubule localization, and *oskar* mRNA localization and translation. *Development*. 129: 517-26.

Duffy, J. B., Perrimon, N. 1994. The torso pathway in Drosophila: lessons on receptor tyrosine kinase signalling and pattern formation. *Dev Biol.* 166:380-395.

Ephrussi, A., L. K. Dickinson, and R. Lehmann. 1991. *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell.* 66:37-50.

Ephrussi, A., and R. Lehmann. 1992. Induction of germ cell formation by *oskar*. *Nature.* 358:387-392.

Erdélyl, M., A.-M. Michon, A. Guichet, J. B. Glotzer, and A. Ephrussi. 1995. Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. *Nature.* 377:524-527.

Grether, M. E., Abrams, J.M., Agapite, J., White, K., Steller, H. 1995. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes & Dev.* 9:1694-1708.

Hanyu-Nakamura, K., Kobayashi, S., Nakamura, A. 2004. Germ-cell autonomous Wunen2 is required for germline development in Drosophila embryos. *Development.* 131:4545-4553.

Hay, B., L. Ackerman, S. Barbel, L. Jan, and Y. N. Jan. 1988. Identification of a component of *Drosophila* polar granules. *Development.* 103:625-640.

Hou, X. S., Chou, T.B., Melnick, M.B., Perrimon, N. 1995. The torso receptor tyrosine kinase can activate Raf in a Ras-independent pathway. *Cell.* 81:63-71.

Jaglarz, M. K., Howard, K.R. 1994. Primordial germ cell migration in Drosophila melanogaster is controlled by somatic tissue. *Development.* 120:83-89.

Jaglarz, M. K., and K. R. Howard. 1995. The active migration of *Drosophila* primordial germ cells. *Development.* 121:3495-3503.

Jankovics, F., R. Sinka, T. Lukacsovich, and M. Erdelyi. 2002. MOESIN Crosslinks Actin and Cell Membrane in Drosophila Oocytes and Is Required for OSKAR Anchoring. *Curr Biol.* 12:2060-2065.

Jones, M.C., Caswell, P.T., Norman, J.C. 2006. Endocytic recycling pathways: emerging regulators of cell migration. *Curr Opin Cell Biol*. 18:549-57.

Kim-Ha, J., J. L. Smith, and P. M. Macdonald. 1991. *oskar* mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell.* 66:23-35.

Kim-Ha, J., K. Kerr, and P. M. Macdonald. 1995. Translational regulation of *oskar* mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell.* 81:403-412.

Klinger, M., M. Erdelyi, J. Szabad, and C. Nüsslein-Volhard. 1988. Function of *torso* in determining the terminal anlagen of the *Drosophila* embryo. *Nature.* 335:275-277.

Kunwar, P. S., Starz-Gaiano, M., Bainton, R.J., Heberlein, U., Lehmann, R. 2003. Tre1, a G-protein-coupled receptor, directs transepithelial migration of Drosophila germ cells. *PloS Biol.* 3:E80.

Lehmann, R., and C. Nüsslein-Volhard. 1986. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in Drosophila. *Cell.* 47:141-152.

Li, J., Xia, F., Li, W.X. 2003. Coactivation of STAT and Ras is required for germ cell proliferation and invasive migration in Drosophila. *Dev Cell.* 5:787-798.

Li, K., and T. C. Kaufman. 1996. The homeotic target gene centrosomin encodes an essential centrosomal component. *Cell.* 85:585-596.

Macdonald, P. M., P. Ingham, and G. Struhl. 1986. Isolation, structure and expression of *even-skipped*: A second pair-rule gene of Drosophila containing a homeobox. *Cell.* 47:721-734.

Macdonald, P. M., S. K.-S. Luk, and M. Kilpatrick. 1991. Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes Dev.* 5:2455-2466.

Mills, J. C., Stone, N.L., Erhardt, J., Pittman, R.N. 1998. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J. Cell Biol.* 140:627-636.

Moore, L. A., H. T. Broihier, M. Van Doren, L. B. Lunsford, and R. Lehmann. 1998. Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila. *Development.* 125:667-678.

Nagase, T., Nakayama, M., Nakajima, D., Kikuno, R., Ohara, O. 2001. Prediction of the coding sequences of unidentified human genes. XX. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res.* 8:85-95.

Parks, A. L., K. R. Cook, M. Belvin, N. A. Dompe, R. Fawcett, K. Huppert, L. R. Tan, C. G. Winter, K. P. Bogart, J. E. Deal, M. E. Deal-Herr, D. Grant, M. Marcinko, W. Y. Miyazaki, S. Robertson, K. J. Shaw, M. Tabios, V. Vysotskaia, L. Zhao, R. S. Andrade, K. A. Edgar, E. Howie, K. Killpack, B. Milash, A. Norton, D. Thao, K. Whittaker, M. A. Winner, L. Friedman, J. Margolis, M. A. Singer, C. Kopczynski, D. Curtis, T. C. Kaufman, G. D. Plowman, G. Duyk, and H. L. Francis-Lang. 2004. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat Genet.* 36:288-292.

Pelissier, A., Chauvin, J.-P., Lecuit, T. 2003. Trafficking through Rab11 endosomes is required for cellularization during Drosophila embryogenesis. *Curr Biol*. 13: 1848-57.

Pirotta, V. 1988. Vectors for P-mediated transformation in Drosophila. *In* Vectors: A survey of molecular cloning vectors and their uses, Rodriguez, R. L., and D. T. Denhardt, editors. Butterworths, Boston. 437-456.

Polesello, C., I. Delon, P. Valenti, P. Ferrer, and F. Payre. 2002. Dmoesin controls actin-based cell shape and polarity during Drosophila melanogaster oogenesis. *Nat Cell Biol.* 4:782-789.

Raff, J. W., Kellogg, D.R., Alberts, B.M. 1993. Drosophila gamma-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J Cell Biol.* 121:823-835.

Renault, A. D., Y. J. Sigal, A. J. Morris, and R. Lehmann. 2004. Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science.* 305:1963-1966.

Riparbelli, M. G., Callaini, G., Schejter, E.D. 2007. Microtubule-dependent organization of subcortical microfilaments in the early Drosophila embryo. *Dev Dyn.* 236:662-670.

Rongo, C., E. R. Gavis, and R. Lehmann. 1995. Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development.* 121:2737-2746.

Rorth, P. 1998. Gal4 in the Drosophila female germline. *Mech Dev.* 78:113-118.

Sano, H., Renault, A.D., Lehmann, R. 2005. Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen2. *J Cell Biol.* 171:675-683.

Santos, A. C., Lehmann, R. 2004a. Germ cell specification and migration in Drosophila and beyond. *Curr Biol.* 14:R578-89.

Santos, A. C., Lehmann, R. 2004b. Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Dev Cell.* 6:283-293.

Saraste, A., Pulkki, K. 2000. Morphologic and biochemical hallmarks of apoptosis,. *Cardiovasc Res.* 45:528-537.

Seto, M. H., Liu, H.L., Zajchowski, D.A., Whitlow, M. 1999. Protein fold analysis of the B30.2-like domain. *Proteins.* 35:235-249.

Silver, D.L., Montell, D.J. 2001 Paracrine signaling through the JAK/STAT pathway activates invasive behavior of ovarian epithelial cells in *Drosophila*. Cell. 107:831-41.

Smith, J. L., J. E. Wilson, and P. M. Macdonald. 1992. Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. *Cell.* 70:849-859.

Sonnenblick, B. P. 1941. Germ cell movements and sex differentiation of the gonads in the Drosophila embryo. *Proc Natl Acad Sci U S A.* 27:484-489.

Starz-Gaiano, M., N. K. Cho, A. Forbes, and R. Lehmann. 2001. Spatially restricted activity of a Drosophila lipid phosphatase guides migrating germ cells. *Development.* 128:983-991.

Tetzlaff, M. T., Jackle, H., Pankratz, M.J. 1996. Lack of Drosophila cytoskeletal tropomyosin affects head morphogenesis and the accumulation of oskar mRNA required for germ cell formation. *EMBO J.* 15:1247-1254.

Underwood, E. M., J. H. Caulton, C. D. Allis, and A. P. Mahowald. 1980. Developmental fate of pole cells in Drosophila melanogaster. *Dev Biol.* 77:303-314.

Van Doren, M., A. L. Williamson, and R. Lehmann. 1998. Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol.* 8:243-246.

Vanzo, N. F., and A. Ephrussi. 2002. Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. *Development.* 129:3705-3714.

Vanzo, N.F., Oprins, A., Xanthakis, D., Ephrussi, A., Rabouille, C. 2007. Stimulation of endocytosis and actin dynamics by Oskar polarizes the *Drosophila* oocyte. Dev *Cell*. 12: 543-55.

von Heijne, G. 1992. Membrane protein structure prediction: Hydrophobicity analysis and the 'Positive Inside' rule. *J. Mol. Biol.* 225:487-494.

Webster, P. J., J. Suen, and P. M. Macdonald. 1994. *Drosophila virilis oskar* transgenes direct body patterning but not pole cell formation or maintenance of mRNA localization in *D. melanogaster*. *Development.* 120:2027-2037.

Woo, J. S., Imm J.H., Min, C.K., Kim, K.J., Cha, S.S., Oh, B.H. 2006. Structural and functional insights in the B30.2/SPRY domain. *EMBO J.* 25:1353-1363.

Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C.J., Boulianne, G.L. 2001. Neuralized functions as an E3 ubiquitin ligase during Drosophila development. *Curr Biol.* 11:1675-1679.

Yu, S. Y., Yoo, S.J., Yang, L., Zapata, C., Srinivasan, A., Hay, B.A., Baker, N.E. 2002. A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye. *Development.* 192:3269-3278.

Zhang, N., J. Zhang, K. J. Purcell, Y. Cheng, and K. Howard. 1997. The Drosophila protein Wunen repels migrating germ cells. *Nature.* 385:64-67.

Zhang, N., Zhang, J., Cheng, Y., Howard, K. 1996. Identification and genetic analysis of wunen, a gene guiding Drosophila melanogaster germ cell migration. *Genetics.* 143:1231-1241.

Appendix A: Partial purification of the polar granules of *Drosophila melanogaster*

ABSTRACT

The germ cells of *Drosophila melanogaster* are set aside early in embryogenesis, and are unique in the embryo in that they contain large (~0.7 μm) ribonucleoprotein complexes called polar granules. While it is known that the polar granules are necessary for germ cell formation, the exact function of the granules in germ cell specification is unknown. Only five components of the granules have been identified so far (Oskar, Vasa, Tudor, Aubergine and *pgc RNA*). Considering the size of the granules, it is plausible that there are more components that, together with the functions of the known granule components, might better elucidate the function of the granules. In order to isolate further components of the polar granules, I attempted to purify the granules biochemically, using GFP-tagged Aubergine as a marker to detect the granules after various purification steps. Unfortunately, my attempts were not successful. I was only able to follow the granules through two purification steps, and the fractions the granules appear to be in remained substantially impure.

INTRODUCTION

The early localization of cytoplasmic determinants to limited regions within the oocyte is observed in many animals as a way to specify cell fate. In *Drosophila melanogaster*, a specialized cytoplasm called pole plasm is localized to the posterior pole of the oocyte and is required for germ cell formation (for a review, see Mahowald, 2001) Structures known as polar granules assemble from a subset of the components of this cytoplasm.

The pole plasm forms beginning at stage 9 of oogenesis when *oskar* mRNA is localized to the posterior pole and Oskar protein begins to be translated (Kim-Ha et al., 1991, Rongo et al., 1995). Subsequently, Vasa, Aubergine, and Tudor proteins also localize and associate with Oskar protein at the posterior pole (Breitwieser et al., 1996, Ephrussi and Lehmann, 1992, Liang et al., 1994). Small granules (~0.2 μm) are formed in the oocyte that persist at the posterior pole of the freshly laid egg and are incorporated into the forming germ cells (pole cells) of the embryo. They will remain in the pole cells as the embryo begins gastrulation, however they are short-lived. A related structure, called nuage, which shares components and ultrastructural similarity with the granules, will replace the granules as the pole cells complete migration and associate with somatic cells to form the gonad of the larva. Nuage is found perinuclearly in the cells of the gonads throughout the larval and adult stages in the fly (Mahowald, 1971). It is also found in the germ cells of a wide variety of animals, underscoring the importance of this ultrastructural feature of germ cells (Eddy, 1975).

The known protein components of polar granules are members of the *grandchildless* class of maternal effect genes. All have been shown to be essential for formation of polar granules and pole cells, since mothers that are homozygous mutant for any of these genes produce embryos lacking both features (Boswell and Mahowald, 1985, Frohnhofer et al., 1986, Harris and Macdonald, 2001, Schupbach, 1989). Isolation of further members of this class of genes through another *grandchildless* screen is a large undertaking, and would likely only uncover a few more components important for forming polar granules. The biochemical purification of polar granules has been attempted by several labs, including Waring et al. (1978). The authors reported the isolation of a subcellular fraction consisting predominately of polar granules, as

determined by EM analysis. However, the purity of this fraction was not sufficient to permit identification of components of the granules directly.

In order to identify further components of the granules, I attempted to biochemically purify the polar granules of *D. melanogaster*. I used transgenic flies expressing green fluorescent protein-tagged Aubergine (GFP-Aub) as a source of polar granules and detected the granules by fluorescence microscopy. This fluorescence visual assay has proven to be a viable method for determining the effects of lysis buffers on the granules' morphology, as well as detection of the granules in fractions following purification steps. Unfortunately, while known components of the granules could be detected in fractions by western, none of the known components of the granules were detected by mass spectral analysis. Reasons for this failure and possible solutions are discussed in this appendix.

RESULTS

Partial purification of polar granule material

In order to identify further components of the polar granules, I used transgenic flies expressing green fluorescent protein-tagged Aubergine (GFP-Aub Harris and Macdonald, 2001) as a source of embryos. Polar granules from these embryos are easily monitored on slides using the fluorescence microscope, whether in intact embryos or in lysate (Fig A.1C). Using this method for detection of the granules, it is possible to assay the effects of lysis buffers on the granules' morphology, as well as detect the granules in fractions after various purification steps.

0 – 3 hour embryos were used for the purification. Salt tolerance was the first condition that was tested. Using KCl as the salt in buffer containing 50 mM Tris pH 7.5

and 10% glycerol, the granules were able to withstand 200 mM KCl before the size and number of granules seen in each sample began to diminish. Therefore, salt concentrations in all following experiments were kept below 200 mM salt. While testing differential centrifugation as a possible purification step, various detergents were tested for their ability to keep the granules from aggregating. 0.15% NP-40 prevented some aggregation, however the majority granules were still pelleted into a large mass that could not be resuspended, therefore differential centrifugation as a possible step was abandoned.

A Nycodenz density gradient was then tested to separate out the granules from the bulk of free protein while also preventing aggregates containing the granules from forming. Several gradients were run using various buffer conditions, however, 50 mM sodium citrate was the best salt and concentration for keeping the granules intact in the gradient. The final buffer conditions (see materials and methods) allow for sedimentation of the intact granules through the gradient without the use of crosslinking reagents. The granules are detectable in positive fractions by western of known components and by fluorescence microscopy (Fig A.1B,D).

Strong anion and cation exchangers were then tested as a step to follow the gradient, however neither resin bound the granules in the lysis buffer used for the gradient. pH conditions for the granules were then tested, and since the granules could withstand a pH between 4 and 9 without disintegrating, the buffer was fine-tuned to test for more efficient binding and then also elution from Q and SP sepharose separately. The granules from positive fractions in the gradient bound only to the SP sepharose by using a low pH buffer on the column (see materials and methods). The column was then washed in the low pH buffer, and fractions were eluted using a buffer with increased pH and salt conditions (see materials and methods). Using this method, the granules are bound and

can be eluted from the column, however the amount recovered is low as assayed by western (Fig A.2).

Problems maintaining the integrity of the granules became apparent upon scaling up for possible gel filtration to follow the SP sepharose chromatography. The gradient fractionation step was scaled up by adding more lysate per gradient (up to 5% gradient volume) and running several gradients simultaneously. Despite obtaining an apparent increase in the amount of polar granule material in positive fractions from the gradient (as assayed by western), there was no substantial increase in the amount of material containing polar granule components obtained after SP sepharose chromatography. Possible problems with proteolysis became apparent as more highly concentrated material was loaded onto gels for westerns, because aberrant lower molecular weight bands became visible. In addition, the granules would occasionally be associated with larger particulate material in the positive gradient fractions (as assayed by fluorescence microscopy).

To combat these problems, the amount of protease inhibitors included was tested, as well as adjusting the amount of lysate loaded onto each gradient, and the method of preparing the lysate. Protease inhibitor cocktail concentrations from 1 to 10x were tested with no change in the banding pattern on westerns as protease inhibitor concentration increased, therefore 1x concentration was maintained. To dissociate the granules from any particulate matter, a decrease in lysate loaded onto the gradient and sonication of the lysate were tested. Previous gradients had been run with lysate made by dounce homogenization. In order to disrupt any particulate matter that the granules may already be associated with, sonication in additional lysis buffer was performed. Lysate at an effective concentration of 2.5% gradient volume was then loaded. With these

modifications, an increase in the amount of material in SP sepharose elution fractions was obtained according to western.

Mass spectrometry analyses of partially purified fractions

Fractions from the SP sepharose column were analysed by electrospray mass spectral analysis at the UT Austin ICMB Core Facility before the sonication modification of the lysate preparation. From this analysis (Table A1), none of the known components of the granules were detected. Of the proteins identified, the majority were CG genes with only a putative function, and the subcellular localization of these proteins is unknown. Lacking the identification of any of the known components of the granules by this MS analysis, any correlation between identification of these proteins and association with the granules was impossible. Further improvements to the purification were then attempted as stated above.

After the sonication modification, individual bands of the SP sepharose fractions were sent off to the Duke Proteomics Center for analysis by MALDI mass spectrometry. Unfortunately, again, none of the known components were detected (Table A2). In this case, it is possible that the known components were not detected due to errors in estimating the size of the bands. However, no further analysis was carried out on these fractions.

DISCUSSION

The polar granules of *Drosophila melanogaster* are a fascinating structure, and have been studied by many because of their enormous form and unique ability to determine the germ line (Ephrussi and Lehmann, 1992). The molecular form and function

of the granules, however, continues to remain a mystery. Biochemical purification of the granules is an attractive proposal, as it would allow identification of several components at once and also present the possibility of determining the structure of the granules at the molecular level. The large size of the granules and the fact that they appear free in the cell also contributes to the idea that the granules might be straightforward to isolate (Mahowald, 1962).

The isolation of polar granules has been attempted previously. Waring et al. (1978) used differential centrifugation to pellet the granules, and relied on the use of electron microscopy to monitor for the presence of granules after centrifugation. The use of GFP-Aubergine as a marker for the granules in this work greatly facilitated identification of the granules after purification steps. Using this method of detection, I was able to determine that the granules were not only pelleted by differential centrifugation, but also trapped in a mass of cell debris that the granules could not be freed from. I was also able to identify free granules in fractions of the Nycodenz gradient that are separated from the bulk of free protein in the embryo. This one step is not sufficient to purify the granules completely though. The proteins identified by either method of mass spectrometry are in large part proteins associated with dense cell parts, such as microtubules, membranes and nuclei. Several successive Nycodenz density gradients could be required to attempt to progressively separate the granules away from these contaminants. The SP sepharose step of the purification procedure probably did little if anything to further purify the granules. The granules could be bound and eluted from the column according to westerns, but practically no protein is detected in the flow-through or wash fractions. In addition, the amount of polar granule components detected by western is dramatically reduced after the column, and I was unable to detect granules in these fractions by fluorescence microscopy.

Overall, it may be a better strategy to intentionally disrupt the granules using high salt to create a pool of smaller, more similarly sized granules. The polar granules are large in size, up to 50 times bigger than a eukaryotic ribosome, however the size is quite heterogeneous (~ 0.2 – 0.7 μm in diameter in cellular blastoderm stage embryos) preventing sedimentation to a discrete band in Nycodenz or sucrose gradients as has been used to isolate ribosomes (Nieuwenhuysen, 1978, Nieuwenhuysen, 1982). There is evidence from *D. immigrans* embryos that the larger polar granules found in gastrulating embryos might be made of many smaller granules that fuse together. In these embryos, the larger polar granules that form at gastrulation have a crystalline substructure whose repeats are of similar size to the smaller, possible precursor, granules of earlier staged embryos (Mahowald et al., 1976). Therefore the smaller granules seen in lysates containing salt above 250 mM may be individual subunits of the granules, containing the same proteins. These smaller granules might sediment into a more discrete band in a Nycodenz gradient and could then be isolated using an affinity tag. I did attempt to purify the granules from embryos from flies carrying a transgene encoding Aubergine fused to the TAP tag (Rigaut et al., 1999). While it is possible to purify free TAP-Aub using this method, no other granule component co-purifies with TAP-Aub. We also centrifuged lysate from TAP-Aub embryos on a Nycodenz density gradient. While TAP-Aub is detected by western in the fractions corresponding to those which contained granules in previous Nycodenz gradients, TAP-Aub from these fractions cannot be pulled down using the IgG or Calmodulin beads, suggesting that the TAP tag may be obscured in the granules. Perhaps the TAP-tag on another protein component of the granules might work better for this kind of scheme.

MATERIALS AND METHODS

Purification scheme to isolate polar granule material

0-3 hour embryos from P[uas-gfp-aubergine]/TM3 flies (Harris and Macdonald, 2001) were collected in population cages on apple juice agar trays at 25°C. The embryos were dechorionated with bleach and collected and rinsed in 0.1% Triton X-100 using a Millipore filtration device. The embryos were then rinsed in 2x volume of lysate buffer at 4°C (50 mM Sodium Citrate, 50 mM HEPES pH 7.5, 10% Glycerol, 0.15% NP-40, 5mM EDTA, 7mM $MgCl_2$, 1 μg/ ml Pepstatin, 1x Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), DEPC treated) three times, and dounce homogenized using a pellet pestle in 2x volume of lysate buffer at 4°C. Approximately 600μl of lysate (5% gradient volume) was then layered onto each of 2-4, 12mL gradients, made of 5 – 35% Nycodenz (Sigma Chemical Co., St. Louis, MO.) prepared in the lysate buffer. The gradients were spun in a Beckman ultracentrifuge using an SW41Ti rotor at 30.7K rpm for 3 hours at 4°C, max acceleration, no brake. 35 - 350μL fractions were pipetted from the top of the gradient down. The fractions were analysed using the fluorescence microscope for the presence of granules. Peak positive fractions (usually three fractions between 18-24) were pooled and loaded onto a 2 ml SP sepharose column that had previously been rinsed three times in three times bed volume of column start buffer (50 mM Sodium Citrate, 50 mM HEPES pH 5, 10% Glycerol, 1 μg/ ml Pepstatin, 1x Complete Protease Inhibitor cocktail, DEPC treated). The volume loaded was allowed to flow through by gravity flow. The column was then rinsed three times in three times bed volume of the start buffer. Protein was then eluted from the column using higher salt and higher pH elution buffer (200 mM sodium citrate, 50 mM HEPES pH 9, 10% glycerol, 1

μg/ ml pepstatin, 1x complete protease inhibitor cocktail, DEPC treated). 300 μL fractions were collected by gravity flow.

Sonication modification: Embryos were collected and rinsed as described above, however the embryos were sonicated in 2X volume of lysate buffer at 4°C using a Branson Sonifier 250 sonicator set at power level 1, duty cycle 90%. The embryos were sonicated on ice using four 10 second pulses with a five second rest in between each pulse.

Analysis of Nycodenz and SP Sepharose fractions

Fractions from both the Nycodenz gradient and SP sepharose column were analysed using SDS PAGE gel electrophoresis, western blot analysis, and fluorescence analysis using a Nikon E600 fluorescence microscope to detect the presence of the granules. Antibodies were used at the following dilutions: rat α-Vasa, 1:500; and rabbit α-Osk, 1:1000 (a gift from Paul Lasko).

Positive fractions from the SP sepharose column were sent off for mass spectrometry analysis at both the UT Austin ICMB Core Facility and the Duke Proteomics Center. For electrospray mass spectrometry analysis, a single 300μL fraction from the SP column was processed by the UT Austin ICMB Core Facility. For MALDI mass spectrometry, the top three fractions were pooled and separated using SDS PAGE. Strong bands at the following molecular weights (15kD, 45kD, 55kD, 70kD, 80kD, 90kD, 160kD, 175kD, 230kD) were excised and stored at 70°C before being sent off. The apparent molecular weights of the known granule components are as follows: Oskar, 55 and 71 kD; Vasa, 70kD; Aubergine, 105kD; and Tudor 135 kD, 205 kD and 285 kD.

ACKNOWLEDGEMENTS

I would like to thank Paul Macdonald and Eric Arn for discussions and help with each step of the purification procedure. I would also like to thank Klause Linse and the Duke Proteomics Center for processing of the Mass Spectrometry samples and help in interpretation of the data. Thanks also to Paul Lasko for the gift of the α-Oskar antibody.

TABLES

Table A1: Results[a] from electrospray mass spectrometry analysis of positive fractions from SP column chromatography.

Ion score[b]	Protein name/ identifier	Biological process	Function inferred from electronic annotation only?	Subcellular localization
87	Bellwether	ATP synthesis coupled proton transport	Yes	Mitochondrial membrane
83	CG5789	Response to toxin	Yes	Integral to membrane
62	Herc2	Mitosis	Yes	Intracellular
61	CG1842	Microtubule-based movement	Yes	Microtubule
60	CG11198	Fatty acid biosynthesis	Yes	Unknown
56	Highwire	Locomotion, BMP signaling	No	Plasma membrane
55	CG14438	Nucleic acid binding	Yes	Unknown
53	Mip130	Neg. reg. of transcription from RNA pol II	Yes	Nucleus
53	CG30069	Cell cycle control	Yes	Unknown
50	CG15899	Calcium ion transport	Yes	Integral to membrane
50	Split ends	Wnt receptor signaling pathway	Yes	Nucleus
49	Mei-41	Cell cycle checkpoint	No	Unknown
49	GEF64C	Axon guidance	No	Intracellular
48	CG12139	Calcium binding, receptor mediated endocytosis	Yes	Membrane
47	CG4038	SnoRNA binding	Yes	SnRNA complex
46	Suppressor of Sable	mRNA binding	Yes	Nucleus
46	CG14395	Unknown	N/A	Unknown
45	Futsch	Microtubule binding	No	Microtubule
45	Germline	DNA binding, DNA ligase	Yes	Nucleus

	transcription factor 1	activity		
45	Smg5	Nonsense mediated decay	No	Unknown
45	CG2989	Chitin binding, cell signaling	Yes	Extracellular
45	Vha100-1	Cation transport	Yes	Hydrogen-transporting ATPase V0 domain
44	Eps15	Calcium ion binding, endocytosis	Yes	Unknown
43	Fat2	Cell adhesion molecule	Yes	Integral to membrane
43	NotI	Negative regulator of basal transcription	Yes	Unknown
43	East	Carboxypeptidase A activity	Yes	Nucleus
42	Micropia	Unknown	N/A	Unknown
42	CG33087	ATP binding	Yes	Membrane
41	CG3585	Receptor binding, exocytosis	Yes	Microtubule
40	CG8478	Nucleic acid binding	Yes	Unknown
39	Dre4	Transcription cofactor	Yes	Nucleus
39	CG17593	Unknown	N/A	Unknown
39	InaD	Calmodulin binding	No	Integral to membrane
38	CG30263	Unknown	N/A	Unknown
38	Mutator 2	Unknown	N/A	Unknown
38	CG10966	Diacylglycerol binding	No	Membrane
37	EIF3-S10	Translation initiation factor	Yes	Cytoplasm
37	Klumpfuss	Nucleic acid binding	Yes	Nucleus
37	Neuro-fibromin1	Ras GTPase activator activity	No	Unknown
37	Bluestreak	Oskar mRNA localization	No	Unknown
36	CG11847	Unknown	N/A	Unknown
35	CG5486	Ubiquitin specific protease	Yes	Nucleus
35	CG5205	ATP binding	Yes	SnRNP complex
35	CG11122	Nucleic acid binding	Yes	Unknown
35	CG6686	Transcription regulator activity	Yes	Unknown
35	CG30007	Unknown	N/A	Unknown
35	α-Est2	Carboxylesterase activity	Yes	Unknown
34	Rictor	Unknown	N/A	Unknown
34	Mei-218	Meiotic recombination	Yes	Cytoplasm
34	Ddp-1	DNA binding	No	Nucleus
34	CG8828	Unknown	N/A	Unknown
34	CG17150	Microtubule motor activity	Yes	Microtubule
34	Pms2	ATP binding	Yes	Unknown
34	CG13503	Unknown	N/A	Unknown
34	Rudimentary	ATP binding	Yes	Cytoplasm
33	CG4790	Unknown, terminal region determination	No	Extracellular
33	Pol	Unknown	N/A	Unknown
33	GAG-like	Unknown	N/A	Unknown
33	Furin 2	ATP binding, Furin activity	Yes	Plasma membrane
33	CG11321	Protein binding	Yes	Unknown

[a] Results were originally obtained as a peptide report listing protein name, ion score, mass and peptides matched. Information concerning function and subcellular localization were obtained from the most current version of Flybase (December 8, 2006 version, http://flybase.bio.indiana.edu/).

[b] Individual ion scores above 37 indicate identity or extensive homology.

Table A2: Results[a] from MALDI mass spectrometry analysis of positive fractions from SP column chromatography.

Size of band cut out	Candidate proteins	Molecular weight of candidate protein (kD)	Biological process	Function inferred from electronic annotation only?	Subcellular localization
p15	CG13081	19.7	Unknown	N/A	Unknown
p45	Ef1α48D	50.3	Translation elongation	Yes	Cytoplasm
	Ef1α100E	50.6	Translation elongation	Yes	Cytoplasm
	Yp3	46.7	Vitellogenesis	Yes	Unknown
	Yp1	48.7	Vitellogenesis	Yes	Unknown
P55	ATP-syn B	54.1	Cell motility	Yes	ATP synthase complex
	Bellwether	59.4	ATP synthesis coupled proton transport	Yes	Mitochondrial membrane
	CG6572	62.7	Unknown	N/A	Unknown
P70	Hsp60	60.7	Protein folding, response to heat	Yes	Mitochondrion
	Zw alt1221	60.4	Pentose-phosphate shunt	No	Unknown
P80	Hsc70-4	71.0	Protein folding	Yes	Mitochondrion, nucleus
P90	CG7343	80.1	Positive regulation of body size	Yes	Membrane, nucleus
P175	Bluestreak	194.4	Oskar mRNA localization	No	Unknown

[a] Results were originally obtained as a peptide report listing protein name, mass and peptide count. Information concerning function and subcellular localization were obtained from the most current version of Flybase (December 8, 2006 version, http://flybase.bio.indiana.edu/).

FIGURES

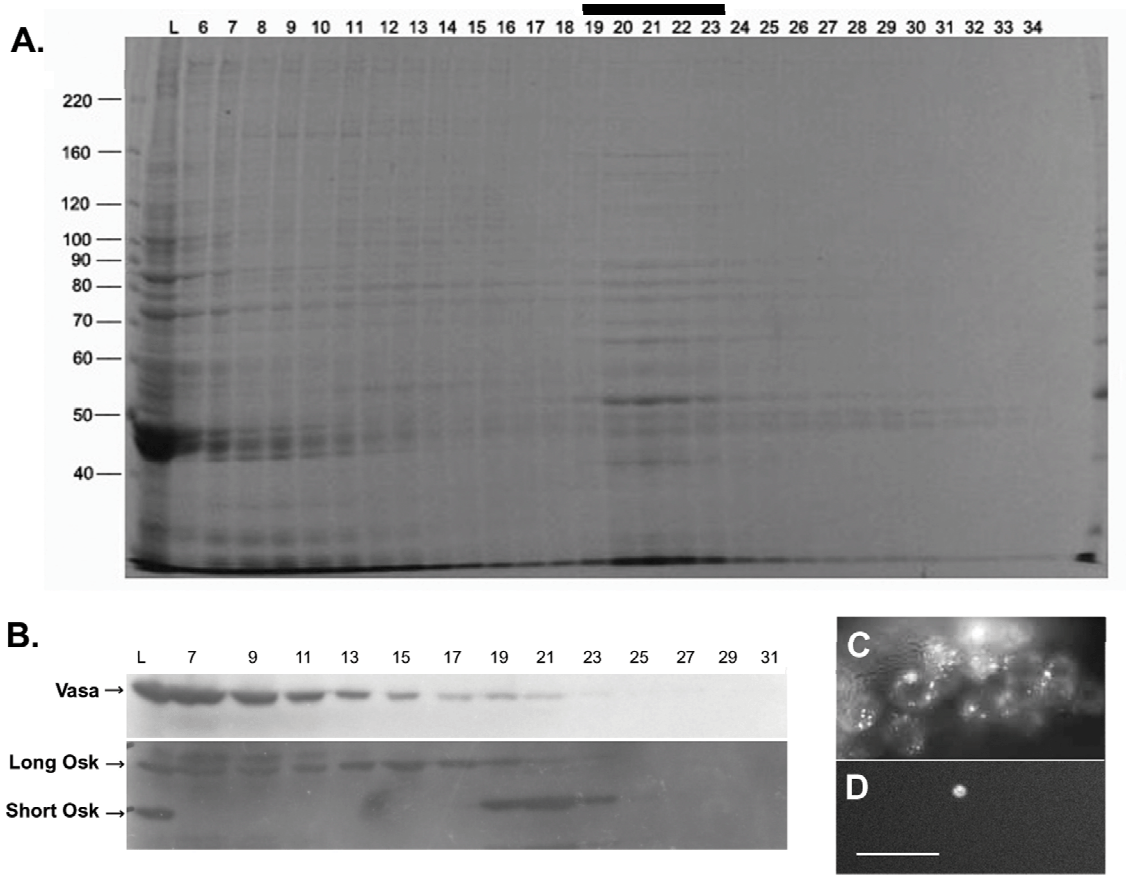


Figure A.1: SDS PAGE, western analysis, and fluorescence detection of polar granules in fractions from the Nycodenz gradient.

A. Coomassie stained gel of fractions from the Nycodenz gradient. Lanes on the gel from left to right correspond to fractions from the top to the bottom of the gradient. Fractions 19-23 (marked by the black bar) correspond to those fractions in which granules could be detected by fluorescence microscopy. L, lysate. B. Western analysis of Nycodenz gradient fractions. Both Vasa and Oskar proteins are detected in the same fractions that were positive for polar granules according to fluorescence microscopy. C. and D. GFP-Aub granules are detectable by fluorescence microscopy both in intact embryos (C) and in fractions from the gradient (D).

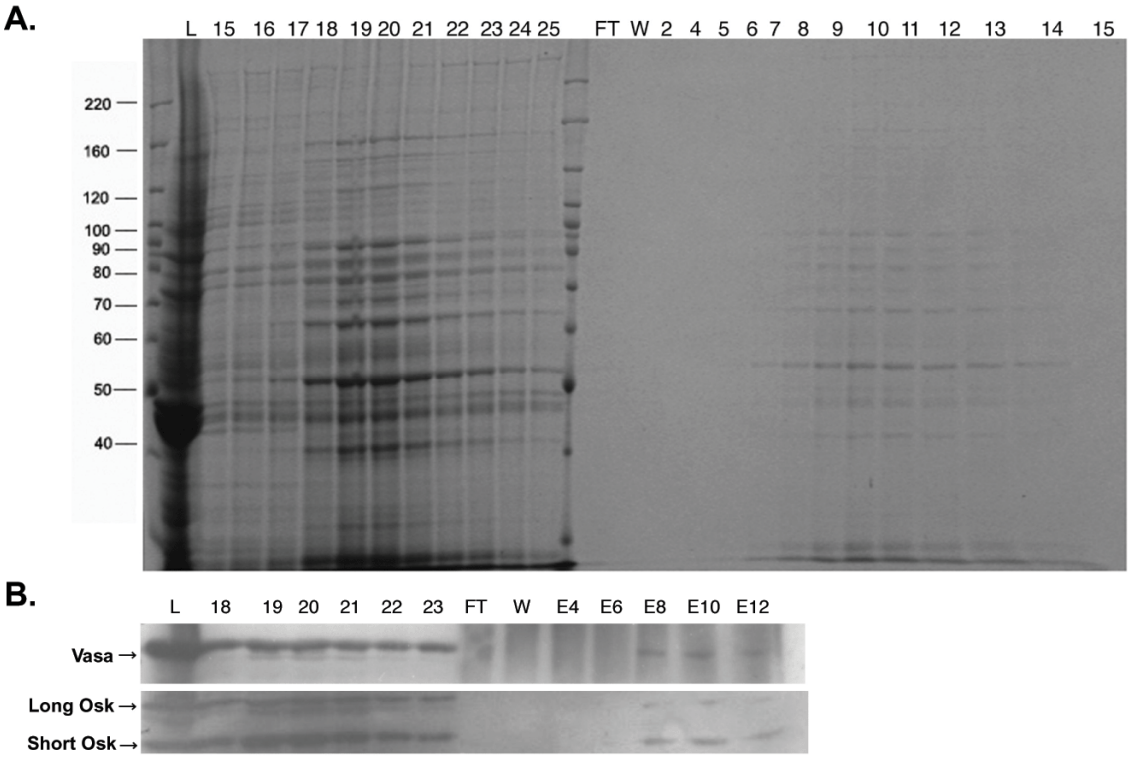


Figure A.2: SDS PAGE and Western analysis of fractions from both the Nycodenz gradient and SP sepharose column.

A. Coomassie stained gel of positive fractions from the gradient as well as fractions from the SP sepharose column before scale up. L lysate; FT, flow through; W, wash. B. Western analysis of positive fractions from the Nycodenz column and the flow-through, wash and selected fractions of the SP sepharose column. Both Oskar and Vasa are detectable in fractions from the gradient as well as the column.

REFERENCES

Boswell, R. E., and A. P. Mahowald. 1985. *tudor*, a gene required for assembly of the germ plasm in Drosophila melanogaster. *Cell.* 43:97-104.

Breitwieser, W., F.-H. Markussen, H. Horstmann, and A. Ephrussi. 1996. Oskar protein interaction with vasa represents an essential step in polar granule assembly. *Genes Dev.* 10:2179-2188.

Eddy, E. M. 1975. Germ plasm and the differentiation of the germ cell line. *Int. Rev. Cytol.* 43:229-280.

Ephrussi, A., and R. Lehmann. 1992. Induction of germ cell formation by *oskar*. *Nature.* 358:387-392.

Frohnhofer, H. G., R. Lehmann, and C. Nusslein-Volhard. 1986. Manipulating the anteroposterior pattern of the Drosophila embryo. *J Embryol Exp Morphol.* 97 Suppl:169-179.

Harris, A. N., and P. M. Macdonald. 2001. Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development.* 128:2823-2832.

Kim-Ha, J., J. L. Smith, and P. M. Macdonald. 1991. *oskar* mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell.* 66:23-35.

Liang, L., W. Diehl-Jones, and P. Lasko. 1994. Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. *Development.* 120:1201-1211.

Mahowald, A. P. 2001. Assembly of the Drosophila germ plasm. *Int Rev Cytol.* 203:187-213.

Mahowald, A. P., K. Illmensee, and F. R. Turner. 1976. Interspecific transplantation of polar plasm between Drosophila embryos. *J Cell Biol.* 70:358-373.

Mahowald, A. P. 1962. Fine structure of pole cells and polar granules in *Drosophila melanogaster*. *J. exp. Zool.* 151:201-216.

Mahowald, A. P. 1971. Polar granules of *Drosophila*. III. The continuity of polar granules during the life cycle of *Drosophila*. *J. exp. Zool.* 176:329-344.

Nieuwenhuysen, P., Clauwaert, J. 1982. Ribosome purification by isopycnic sedimentation in high density sucrose gradients. *Prep Biochem.* 12:229-234.

Nieuwenhuysen, P., Slegers, H. 1978. Purification of eukaryotic ribosomes by isopycnic centrifugation in sucrose. *Anal Biochem.* 89:472-480.

Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol.* 17:1030-1032.

Rongo, C., E. R. Gavis, and R. Lehmann. 1995. Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development.* 121:2737-2746.

Schupbach, T., Weischaus, E. 1989. Female sterile mutations on the second chromosome of Drosophila melanogaster. I. Maternal effect mutations. *Genetics.* 121:101-117.

Waring, G. L., C. D. Allis, and A. P. Mahowald. 1978. Isolation of polar granules and the identification of polar granule-specific protein. *Dev. Biol.* 66:197-206.

Bibliography

Aravin, A. A., Klenov, M., Vagin, V.V., Bantignies, F., Cavalli, G., Gvozdev, V.A. 2004. Dissection of a natural RNA silencing process in the *Drosophila melanogaster* germ line. *Mol Cell Biol.* 24:6742-6750.

Arkov, A. L., J. Y. Wang, A. Ramos, and R. Lehmann. 2006. The role of Tudor domains in germline development and polar granule architecture. *Development.*

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K., Kobayashi, S. 1999. Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. *Nat Cell Biol.* 1:431-437.

Bardsley, A., K. McDonald, and R. E. Boswell. 1993. Distribution of tudor protein in the *Drosophila* embryo suggests separation of functions based on site of localization. *Development.* 119:207-219.

Bellen, H. J., R. W. Levis, G. Liao, Y. He, J. W. Carlson, G. Tsang, M. Evans-Holm, P. R. Hiesinger, K. L. Schulze, G. M. Rubin, R. A. Hoskins, and A. C. Spradling. 2004. The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics.* 167:761-781.

Bergsten, S. E., and E. R. Gavis. 1999. Role for mRNA localization in translational activation but not spatial restriction of *nanos* RNA. *Development.* 126:659-669.

Boswell, R. E., and A. P. Mahowald. 1985. *tudor*, a gene required for assembly of the germ plasm in Drosophila melanogaster. *Cell.* 43:97-104.

Breitwieser, W., F.-H. Markussen, H. Horstmann, and A. Ephrussi. 1996. Oskar protein interaction with vasa represents an essential step in polar granule assembly. *Genes Dev.* 10:2179-2188.

Brennecke, J., D. R. Hipfner, A. Stark, R. B. Russell, and S. M. Cohen. 2003. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell.* 113:25-36.

Broihier, H. T., L. A. Moore, M. Van Doren, S. Newman, and R. Lehmann. 1998. zfh-1 is required for germ cell migration and gonadal mesoderm development in Drosophila. *Development.* 125:655-666.

Brown, S., Zeidler, M.P., Hombria, J.E. 2006. JAK/STAT signalling in Drosophila controls cell motility during germ cell migration. *Dev Dyn.* 235:958-966.

Callaini, G., Riparbelli, M.G., Dallai, R. 1995. Pole cell migration through the gut wall of the Drosophila embryo: analysis of cell interactions. *Dev Biol.* 170:365-375.

Campos-Ortega, J. A. a. H., Volker. 1985. The Embryonic Development of Drosophila melanogaster.

Cerutti, L., N. Mian, and A. Bateman. 2000. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the piwi domain. *Trends Biochem Sci.* 25:481-482.

Cha, B. J., L. R. Serbus, B. S. Koppetsch, and W. E. Theurkauf. 2002. Kinesin I-dependent cortical exclusion restricts pole plasm to the oocyte posterior. *Nat Cell Biol.* 4:592-598.

Chiquione, A. D. 1954. The identification, origin and migration of the primordial germ cells of the mouse embryo. *Anat Rec.* 118:135-146.

Christerson, L. B., and D. M. McKearin. 1994. *orb* is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis. *Genes Dev.* 8:614-628.

Clark, I., E. Giniger, H. Ruohola-Baker, L. Y. Jan, and Y. N. Jan. 1994. Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr. Biol.* 4:289-300.

Claros, M. G. a. v. H., G. 1994. TopPred II: An improved software for membrane protein structure predictions. *CABIOS.* 10:685-686.

Coffman, C. R., Strohm, R.C., Oakley, F.D., Yamada, Y., Przychodzin, D., Boswell, R.E. 2002. Identification of X-linked genes required for migration and programmed cell death of Drosophila melanogaster germ cells. *Genetics.* 126:273-284.

Commisso, C., Boulianne, G.L. 2007. The NHR1 domain of Neuralized binds Delta and mediates Delta trafficking and Notch signalling. *Mol Biol Cell.* 18:1-13.

Counce, S. J. 1963. Developmental morphology of polar granules in Drosophila including observations on pole cell behavior and distribution during embryogenesis. *J. Morph.* 112:129-145.

Deshpande, G., Calhoun, G., Schedl, P. 2004. Overlapping mechanisms function to establish transcriptional quiescence in the embryonic Drosophila germline. *Development.* 131:1247-1257.

Deshpande, G., Swanhart, L., Chiang, P., Schedl, P. 2001. Hedgehog signalling in germ cell migration. *Cell.* 106:759-769.

Dollar, G., Struckoff, E., Michaud, J., Cohen, R.S. 2002. Rab11 polarization of the *Drosophila* oocyte: a novel link between membrane trafficking, microtubule localization, and *oskar* mRNA localization and translation. *Development*. 129: 517-26.

Duffy, J. B., Perrimon, N. 1994. The torso pathway in Drosophila: lessons on receptor tyrosine kinase signalling and pattern formation. *Dev Biol.* 166:380-395.

Eddy, E. M. 1975. Germ plasm and the differentiation of the germ cell line. *Int. Rev. Cytol.* 43:229-280.

Eddy, E. M. 1996. Origin and migration of primordial germ cells in mammals. *Dev Genet.* 19:287-289.

Ephrussi, A., L. K. Dickinson, and R. Lehmann. 1991. *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell.* 66:37-50.

Ephrussi, A., and R. Lehmann. 1992. Induction of germ cell formation by *oskar*. *Nature.* 358:387-392.

Erdélyl, M., A.-M. Michon, A. Guichet, J. B. Glotzer, and A. Ephrussi. 1995. Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. *Nature.* 377:524-527.

Extavour, C. G., and M. Akam. 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development.* 130:5869-5884.

Forbes, A., and R. Lehmann. 1999. Cell migration in Drosophila. *Curr Opin Genet Dev.* 9:473-478.

Frohnhofer, H. G., R. Lehmann, and C. Nusslein-Volhard. 1986. Manipulating the anteroposterior pattern of the Drosophila embryo. *J Embryol Exp Morphol.* 97 Suppl:169-179.

Gavis, E. R., and R. Lehmann. 1994. Translational regulation of *nanos* by RNA localization. *Nature.* 369:315-318.

Gavis, E. R., L. Lunsford, S. E. Bergsten, and R. Lehmann. 1996. A conserved 90 nucleotide element mediates translational repression of *nanos* RNA. *Development.* 122:2791-2800.

Geigy, R. 1931. Action de l'ultra-violet sur le pole germinale dans l'oeuf de Drosophila melanogaster. *Rev. Suisse Zool.* 38:187-288.

Godin, I., Wylie, C., Heasman, J. 1990. Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture. *Development.* 108:357-363.

Gomperts, M., Wylie, C., Heasman, J. 1994. Primordial germ cell migration. *Ciba Foundation Symposium.* 182:121-139.

Grether, M. E., Abrams, J.M., Agapite, J., White, K., Steller, H. 1995. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes & Dev.* 9:1694-1708.

Gunkel, N., T. Yano, F. H. Markussen, L. C. Olsen, and A. Ephrussi. 1998. Localization-dependent translation requires a functional interaction between the 5' and 3' ends of oskar mRNA. *Genes Dev.* 12:1652-1664.

Hanyu-Nakamura, K., Kobayashi, S., Nakamura, A. 2004. Germ-cell autonomous Wunen2 is required for germline development in Drosophila embryos. *Development.* 131:4545-4553.

Harris, A. N., and P. M. Macdonald. 2001. Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development.* 128:2823-2832.

Hay, B., L. Ackerman, S. Barbel, L. Jan, and Y. N. Jan. 1988a. Identification of a component of *Drosophila* polar granules. *Development.* 103:625-640.

Hay, B., L. Y. Jan, and Y. N. Jan. 1988b. A protein component of *Drosophila* polar granules is encoded by *vasa* and has extensive sequence similarity to ATP-dependent helicases. *Cell.* 55:577-587.

Hegner, R. W. 1914. The Germ-Cell Cycle in Animals.

Hou, X. S., Chou, T.B., Melnick, M.B., Perrimon, N. 1995. The torso receptor tyrosine kinase can activate Raf in a Ras-independent pathway. *Cell.* 81:63-71.

Illmensee, K., and A. P. Mahowald. 1974. Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. *Proc. Natl. Acad. Sci. USA.* 71:1016-1020.

Illmensee, K., and A. P. Mahowald. 1976. The autonomous function of germ plasm in a somatic region of the *Drosophila* egg. *Exp. Cell Res.* 97:127-140.

Jaglarz, M. K., Howard, K.R. 1994. Primordial germ cell migration in Drosophila melanogaster is controlled by somatic tissue. *Development.* 120:83-89.

Jaglarz, M. K., and K. R. Howard. 1995. The active migration of *Drosophila* primordial germ cells. *Development.* 121:3495-3503.

Jankovics, F., R. Sinka, T. Lukacsovich, and M. Erdelyi. 2002. MOESIN Crosslinks Actin and Cell Membrane in Drosophila Oocytes and Is Required for OSKAR Anchoring. *Curr Biol.* 12:2060-2065.

Jones, M.C., Caswell, P.T., Norman, J.C. 2006. Endocytic recycling pathways: emerging regulators of cell migration. *Curr Opin Cell Biol*. 18:549-57.

Jongens, T. A., L. D. Ackerman, J. R. Swedlow, L. Y. Jan, and Y. N. Jan. 1994. *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Dev.* 8:2123-2136.

Kashikawa, M., R. Amikura, and S. Kobayashi. 2001. Mitochondrial small ribosomal RNA is a component of germinal granules in Xenopus embryos. *Mech Dev.* 101:71-77.

Kennerdell, J. R., S. Yamaguchi, and R. W. Carthew. 2002. RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. *Genes Dev.* 16:1884-1889.

Kim-Ha, J., J. L. Smith, and P. M. Macdonald. 1991. *oskar* mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell.* 66:23-35.

Kim-Ha, J., K. Kerr, and P. M. Macdonald. 1995. Translational regulation of *oskar* mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell.* 81:403-412.

Kim-Ha, J., P. J. Webster, J. L. Smith, and P. M. Macdonald. 1993. Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development.* 119:169-178.

Kim, J., Daniel, J., Espejo, A., Lake, A., Krishna, M., Xia, L., Zhang, Y., Bedford, M.T. 2006. Tudor, MBT and chromo domains guage the degree of lysine methylation. *EMBO J.* 7:397-403.

King, R. C. 1970. Ovarian development in *Drosophila melanogaste*r.

Klinger, M., M. Erdelyi, J. Szabad, and C. Nüsslein-Volhard. 1988. Function of *torso* in determining the terminal anlagen of the *Drosophila* embryo. *Nature.* 335:275-277.

Knaut, H., F. Pelegri, K. Bohmann, H. Schwarz, and C. Nusslein-Volhard. 2000. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. *J Cell Biol.* 149:875-888.

Kobayashi, S., and M. Okada. 1989. Restoration of pole-cell-forming ability to u.v.-irradiated *Drosophila* embryos by injection of mitochondrial lrRNA. *Development.* 107:733-742.

Kobayashi, S., M. Yamada, M. Asaoka, and T. Kitamura. 1996. Essential role of the posterior morphogen nanos for germline development in *Drosophila*. *Nature.* 380:708-711.

Komiya, T. a. T., Y. 1995. Cloning of a gene of the DEAD box protein family which is specifically expressed in germ cells in rats. *Biochem Biophys Res Commun.* 207:405-410.

Kunwar, P. S., Starz-Gaiano, M., Bainton, R.J., Heberlein, U., Lehmann, R. 2003. Tre1, a G-protein-coupled receptor, directs transepithelial migration of Drosophila germ cells. *PloS Biol.* 3:E80.

Lantz, V., J. S. Chang, J. I. Horabin, D. Bopp, and P. Schedl. 1994. The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* 8:598-613.

Lasko, P. F., and M. Ashburner. 1988. The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. *Nature.* 335:611-617.

Lasko, P. F., and M. Ashburner. 1990. Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. *Genes Dev.* 4:905-921.

Leatherman, J. L., L. Levin, J. Boero, and T. A. Jongens. 2002. germ cell-less acts to repress transcription during the establishment of the Drosophila germ cell lineage. *Curr Biol.* 12:1681-1685.

Lehmann, R., and C. Nüsslein-Volhard. 1986. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in Drosophila. *Cell.* 47:141-152.

Li, J., Xia, F., Li, W.X. 2003. Coactivation of STAT and Ras is required for germ cell proliferation and invasive migration in Drosophila. *Dev Cell.* 5:787-798.

Li, K., and T. C. Kaufman. 1996. The homeotic target gene centrosomin encodes an essential centrosomal component. *Cell.* 85:585-596.

Liang, L., W. Diehl-Jones, and P. Lasko. 1994. Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. *Development.* 120:1201-1211.

Lin, H. 2007. piRNAs in the germ line. *Science.* 316:397.

Macdonald, P. M., P. Ingham, and G. Struhl. 1986. Isolation, structure and expression of *even-skipped*: A second pair-rule gene of Drosophila containing a homeo box. *Cell.* 47:721-734.

Macdonald, P. M., S. K.-S. Luk, and M. Kilpatrick. 1991. Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes Dev.* 5:2455-2466.

Macdonald, P. M., and G. Struhl. 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature.* 324:537-545.

Macdonald, P. M., and G. Struhl. 1988. *Cis*-acting sequences responsible for anterior localization of *bicoid* mRNA in *Drosophila* embryos. *Nature.* 336:595-598.

Mahowald, A. P. 2001. Assembly of the Drosophila germ plasm. *Int Rev Cytol.* 203:187-213.

Mahowald, A. P., K. Illmensee, and F. R. Turner. 1976. Interspecific transplantation of polar plasm between Drosophila embryos. *J Cell Biol.* 70:358-373.

Mahowald, A. P. 1962. Fine structure of pole cells and polar granules in *Drosophila melanogaster*. *J. exp. Zool.* 151:201-216.

Mahowald, A. P. 1968. Polar granules of *Drosophila*. II. Ultrastructural changes during early embryogenesis. *J. exp. Zool.* 167:237-262.

Mahowald, A. P. 1971a. Polar granules of *Drosophila*. III. The continuity of polar granules during the life cycle of *Drosophila*. *J. exp. Zool.* 176:329-344.

Mahowald, A. P. 1971b. Polar granules of *Drosophila*. IV. Cytochemical studies showing loss of RNA from polar granules during early embryogenesis. *J. exp. Zool.* 176:345-352.

Markussen, F.-H., A.-M. Michon, W. Breitwieser, and A. Ephrussi. 1995. Translational control of *oskar* generates Short OSK, the isoform that induces pole plasm assembly. *Development.* 121:3723-3732.

Martinho, R. G., P. S. Kunwar, J. Casanova, and R. Lehmann. 2004. A noncoding RNA is required for the repression of RNApolII-dependent transcription in primordial germ cells. *Curr Biol.* 14:159-165.

Maurer-Stroh, S., Dickens, N.J., Hughes-Davies, L., Kousarides, T., Eisenhaber, F., Ponting, C.P. 2003. The Tudor domain 'Royal Family': Tudor, plant Agenet, Chromo, PWWP and MBT domains. *Trends Biochem Sci.* 28:69-74.

McGinnis, W., M. S. Levine, E. Hafen, A. Kuroiwa, and W. J. Gehring. 1984. A conserved DNA sequence in homoeotic genes of the Drosophila Antennapedia and bithorax complexes. *Nature.* 308:428-433.

Megosh, H. B., D. N. Cox, C. Campbell, and H. Lin. 2006. The Role of PIWI and the miRNA Machinery in Drosophila Germline Determination. *Curr. Biol.* In press.

Mills, J. C., Stone, N.L., Erhardt, J., Pittman, R.N. 1998. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J. Cell Biol.* 140:627-636.

Moore, L. A., H. T. Broihier, M. Van Doren, L. B. Lunsford, and R. Lehmann. 1998. Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila. *Development.* 125:667-678.

Morgan, G. J. 2003. Historical review: Viruses, crystals and geodesic domes. *Trends Biochem Sci.* 28:86-90.

Munro, T. P., S. Kwon, B. J. Schnapp, and D. St Johnston. 2006. A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. *J Cell Biol.* 172:577-588.

Murata, Y., and R. P. Wharton. 1995. Binding of pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. *Cell.* 80:747-756.

Nagase, T., Nakayama, M., Nakajima, D., Kikuno, R., Ohara, O. 2001. Prediction of the coding sequences of unidentified human genes. XX. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res.* 8:85-95.

Nakamura, A., R. Amikura, M. Mukai, S. Kobayashi, and P. F. Lasko. 1996. Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. *Science.* 274:2075-2079.

Newmark, P. A., and R. E. Boswell. 1994. The *mago nashi* locus encodes an essential product required for germ plasm assembly in *Drosophila*. *Development.* 120:1303-1313.

Nieuwenhuysen, P., Clauwaert, J. 1982. Ribosome purification by isopycnic sedimentation in high density sucrose gradients. *Prep Biochem.* 12:229-234.

Nieuwenhuysen, P., Slegers, H. 1978. Purification of eukaryotic ribosomes by isopycnic centrifugation in sucrose. *Anal Biochem.* 89:472-480.

Nieuwkoop, P. D. 1979. Primordial germ cells in the chordates: embryogenesis and phylogenesis.

Parks, A. L., K. R. Cook, M. Belvin, N. A. Dompe, R. Fawcett, K. Huppert, L. R. Tan, C. G. Winter, K. P. Bogart, J. E. Deal, M. E. Deal-Herr, D. Grant, M. Marcinko, W. Y. Miyazaki, S. Robertson, K. J. Shaw, M. Tabios, V. Vysotskaia, L. Zhao, R. S. Andrade, K. A. Edgar, E. Howie, K. Killpack, B. Milash, A. Norton, D. Thao, K.

Whittaker, M. A. Winner, L. Friedman, J. Margolis, M. A. Singer, C. Kopczynski, D. Curtis, T. C. Kaufman, G. D. Plowman, G. Duyk, and H. L. Francis-Lang. 2004. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat Genet.* 36:288-292.

Pelissier, A., Chauvin, J.-P., Lecuit, T. 2003. Trafficking through Rab11 endosomes is required for cellularization during Drosophila embryogenesis. *Curr Biol*. 13: 1848-57.

Pirotta, V. 1988. Vectors for P-mediated transformation in Drosophila. *In* Vectors: A survey of molecular cloning vectors and their uses, Rodriguez, R. L., and D. T. Denhardt, editors. Butterworths, Boston. 437-456.

Polesello, C., I. Delon, P. Valenti, P. Ferrer, and F. Payre. 2002. Dmoesin controls actin-based cell shape and polarity during Drosophila melanogaster oogenesis. *Nat Cell Biol.* 4:782-789.

Poulson, D. F. 1950. Histogenesis, organogenesis and differentiation in the embryo of Drosophila melanogaster. *In: Biology of Drosophila.* 168-274.

Raff, J. W., Glover, D.M. 1989. Centrosomes, not nuclei, initiate pole cell formation in Drosophila embryos. *Cell.* 57:611-619.

Raff, J. W., Kellogg, D.R., Alberts, B.M. 1993. Drosophila gamma-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J Cell Biol.* 121:823-835.

Raz, E. 2000. The function and regulation of vasa-like genes in germ-cell development. *Genome Biol.* 1:REVIEWS1017.

Renault, A. D., Y. J. Sigal, A. J. Morris, and R. Lehmann. 2004. Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science.* 305:1963-1966.

Renault, A. D., Lehmann, R. 2006. Follow the fatty brick road: lipid signaling in cell migration. *Curr Opin Genet Dev.* 16:348-354.

Rhoads, R. E. 1993. Regulation of eukaryotic protein synthesis by initiation factors. *J. Biol. Chem.* 268:3017-3020.

Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol.* 17:1030-1032.

Riparbelli, M. G., Callaini, G., Schejter, E.D. 2007. Microtubule-dependent organization of subcortical microfilaments in the early Drosophila embryo. *Dev Dyn.* 236:662-670.

Rongo, C., E. R. Gavis, and R. Lehmann. 1995. Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development.* 121:2737-2746.

Rongo, C., and R. Lehmann. 1996. Regulated synthesis, transport and assembly of the Drosophila germ plasm. *Trends Genet.* 12:102-109.

Rorth, P. 1998. Gal4 in the Drosophila female germline. *Mech Dev.* 78:113-118.

Saffman, E. E., and P. Lasko. 1999. Germline development in vertebrates and invertebrates. *Cell Mol Life Sci.* 55:1141-1163.

Sano, H., Renault, A.D., Lehmann, R. 2005. Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen2. *J Cell Biol.* 171:675-683.

Santos, A. C., Lehmann, R. 2004a. Germ cell specification and migration in Drosophila and beyond. *Curr Biol.* 14:R578-89.

Santos, A. C., Lehmann, R. 2004b. Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Dev Cell.* 6:283-293.

Saraste, A., Pulkki, K. 2000. Morphologic and biochemical hallmarks of apoptosis,. *Cardiovasc Res.* 45:528-537.

Schüpbach, T., and E. Wieschaus. 1986. Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Dev. Biol.* 195:302-317.

Schupbach, T., Weischaus, E. 1989. Female sterile mutations on the second chromosome of Drosophila melanogaster. I. Maternal effect mutations. *Genetics.* 121:101-117.

Seto, M. H., Liu, H.L., Zajchowski, D.A., Whitlow, M. 1999. Protein fold analysis of the B30.2-like domain. *Proteins.* 35:235-249.

Seydoux, G. a. S., T. 2001. The germline in *C. elegans*: origins, proliferation and silencing. *Int Rev Cytol.* 203:139-185.

Silver, D.L., Montell, D.J. 2001 Paracrine signaling through the JAK/STAT pathway activates invasive behavior of ovarian epithelial cells in *Drosophila*. Cell. 107:831-41.

Smith, J. L., J. E. Wilson, and P. M. Macdonald. 1992. Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. *Cell.* 70:849-859.

Sonnenblick, B. P. 1941. Germ cell movements and sex differentiation of the gonads in the Drosophila embryo. *Proc Natl Acad Sci U S A.* 27:484-489.

Sonnenblick, B. P. 1950. The early embryology of Drosophila melanogaster. *In: Biology of Drosophila.* 168-274.

Spradling, A. C., and G. M. Rubin. 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. *Science.* 218:341-347.

St Johnston, D. 1993. Pole plasm and the posterior group genes. *In* The Development of *Drosophila melanogaster(1)*, Bate, M., and A. M. Arias, editors. Cold Spring Harbor Laboratory Press, New York. 325-363.

Starz-Gaiano, M., N. K. Cho, A. Forbes, and R. Lehmann. 2001. Spatially restricted activity of a Drosophila lipid phosphatase guides migrating germ cells. *Development.* 128:983-991.

Starz-Gaiano, M., and R. Lehmann. 2001. Moving towards the next generation. *Mech Dev.* 105:5-18.

Tautz, D., and C. Pfeifle. 1989. A non radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals a translational control of the segmentation gene hunchback. *Chromosoma.* 98:81-85.

Technau, G. M., and J. A. Campos-Ortega. 1986. Lineage analysis of transplanted individual cells in embryos of *Drosophila melanogaster* III. Commitment and proliferative capabilities of pole cells and midgut progenitors. *Roux's Arch. Dev. Biol.* 195:489-498.

Tetzlaff, M. T., Jackle, H., Pankratz, M.J. 1996. Lack of Drosophila cytoskeletal tropomyosin affects head morphogenesis and the accumulation of oskar mRNA required for germ cell formation. *EMBO J.* 15:1247-1254.

Thomson, T., and P. Lasko. 2004. Drosophila tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. *Genesis.* 40:164-170.

Underwood, E. M., J. H. Caulton, C. D. Allis, and A. P. Mahowald. 1980. Developmental fate of pole cells in Drosophila melanogaster. *Dev Biol.* 77:303-314.

Van Doren, M., A. L. Williamson, and R. Lehmann. 1998. Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol.* 8:243-246.

Vanzo, N. F., and A. Ephrussi. 2002. Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. *Development.* 129:3705-3714.

Vanzo, N.F., Oprins, A., Xanthakis, D., Ephrussi, A., Rabouille, C. 2007. Stimulation of endocytosis and actin dynamics by Oskar polarizes the *Drosophila* oocyte. Dev *Cell*. 12: 543-55.

von Heijne, G. 1992. Membrane protein structure prediction: Hydrophobicity analysis and the 'Positive Inside' rule. *J. Mol. Biol.* 225:487-494.

Waring, G. L., C. D. Allis, and A. P. Mahowald. 1978. Isolation of polar granules and the identification of polar granule-specific protein. *Dev. Biol.* 66:197-206.

Webster, P. J., J. Suen, and P. M. Macdonald. 1994. *Drosophila virilis oskar* transgenes direct body patterning but not pole cell formation or maintenance of mRNA localization in *D. melanogaster*. *Development.* 120:2027-2037.

Wilson, E. B. 1896. The cell in development and inheritance.

Wilson, J. E., J. E. Connell, J. D. Schlenker, and P. M. Macdonald. 1996a. Novel genetic screen for genes involved in posterior body patterning in Drosophila. *Dev Genet.* 19:199-209.

Wilson, J. E., J. E. Connell, and P. M. Macdonald. 1996b. *aubergine* enhances *oskar* translation in the *Drosophila* ovary. *Development.* 122:1631-1639.

Woo, J. S., Imm J.H., Min, C.K., Kim, K.J., Cha, S.S., Oh, B.H. 2006. Structural and functional insights in the B30.2/SPRY domain. *EMBO J.* 25:1353-1363.

Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C.J., Boulianne, G.L. 2001. Neuralized functions as an E3 ubiquitin ligase during Drosophila development. *Curr Biol.* 11:1675-1679.

Yu, S. Y., Yoo, S.J., Yang, L., Zapata, C., Srinivasan, A., Hay, B.A., Baker, N.E. 2002. A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye. *Development.* 192:3269-3278.

Zalokar, M. a. E., I. 1976. Autoradiographic studies of protein and RNA forming during early development of *Drosophila* eggs. *Dev Biol.* 49:425-437.

Zhang, N., J. Zhang, K. J. Purcell, Y. Cheng, and K. Howard. 1997. The Drosophila protein Wunen repels migrating germ cells. *Nature.* 385:64-67.

Zhang, N., Zhang, J., Cheng, Y., Howard, K. 1996. Identification and genetic analysis of wunen, a gene guiding Drosophila melanogaster germ cell migration. *Genetics.* 143:1231-1241.

Vita

Jennifer Rebecca (Hurta) Jones was born in Temple, Texas, on October 12, 1978, to Bernadette and Edward Hurta. She was awarded the degree of Bachelor of Arts in Biology with highest honors from the University of Texas at Austin in May 2000. Jennifer entered the Cell and Molecular Biology graduate program in August of 2000, and joined the Macdonald Lab in May of 2001. During her studies in the lab, she published the following paper:

Jones, J.R. and Macdonald, P.M. (2007). Oskar controls the morphology of polar granules and nuclear bodies in *Drosophila*. *Development.* 134(2): 233-6.

Permanent address: 7208 Trace Chain Drive, Austin, Texas 78749

This dissertation was typed by Jennifer Rebecca Jones.